Assessing the Role of Hypoxia in Tumor Angiogenesis

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Definitions of Hypoxia

- **pO_2 of tissue below "normal" physiological range**
  - **Not a clear distinction between normal and abnormal**
  - **Tissue specific**
- **pO_2 low enough to cause change in radiation effect**
  - ***e.g.* <10 mmHg**

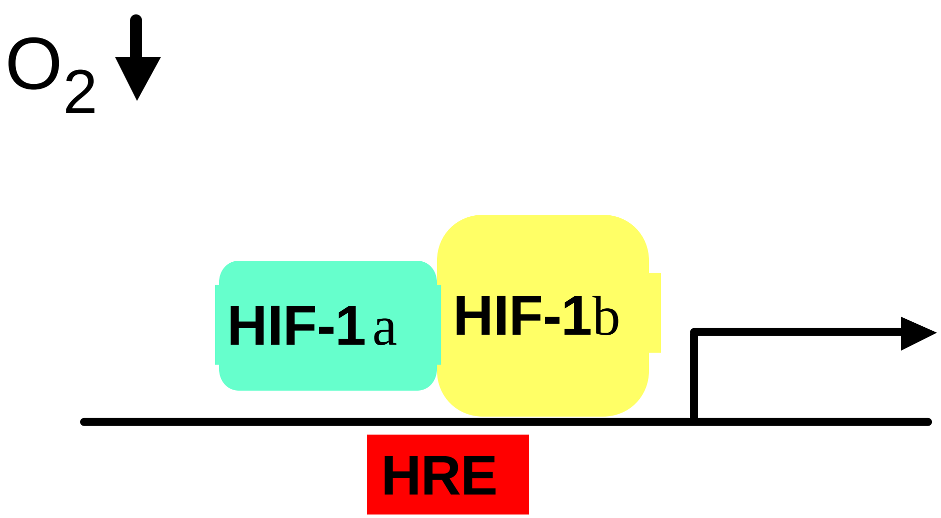
Objectives

- ↑Signal Transduction Pathways
- Hypoxia and angiogenesis in:
  - Normal tissues
  - Pathologic states
  - Tumors
- Conclusions

Promoters Affected by Hypoxia

- **Hif-1**
  - **VEGF, bFGF, iNOS, P53, Glut-1**
- **NFkB**
  - **TNF**a
- **AP-1**
  - **SOD**
- **HSP70**

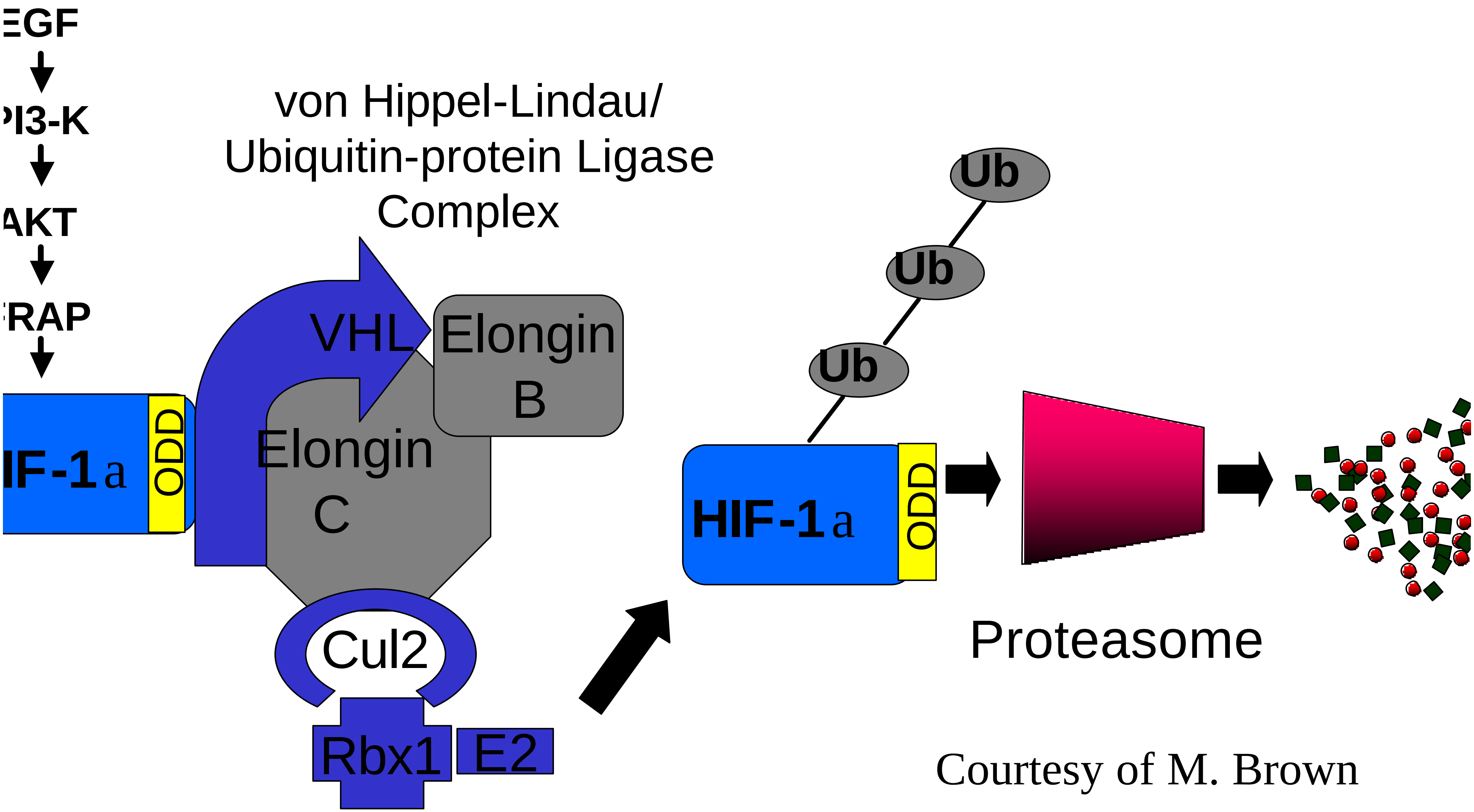
Regulation of Gene Expression under Hypoxia



- **HIF-1**b (**ARNT) is constitutively expressed.**
- **HIF-1**a **levels rise as 02 levels fall.**
- **The** a **and** b **subunits dimerize producing a bHLH-PAS transcription factor that binds t Hypoxia Responsive Elements (HREs) thereby activating genes.**

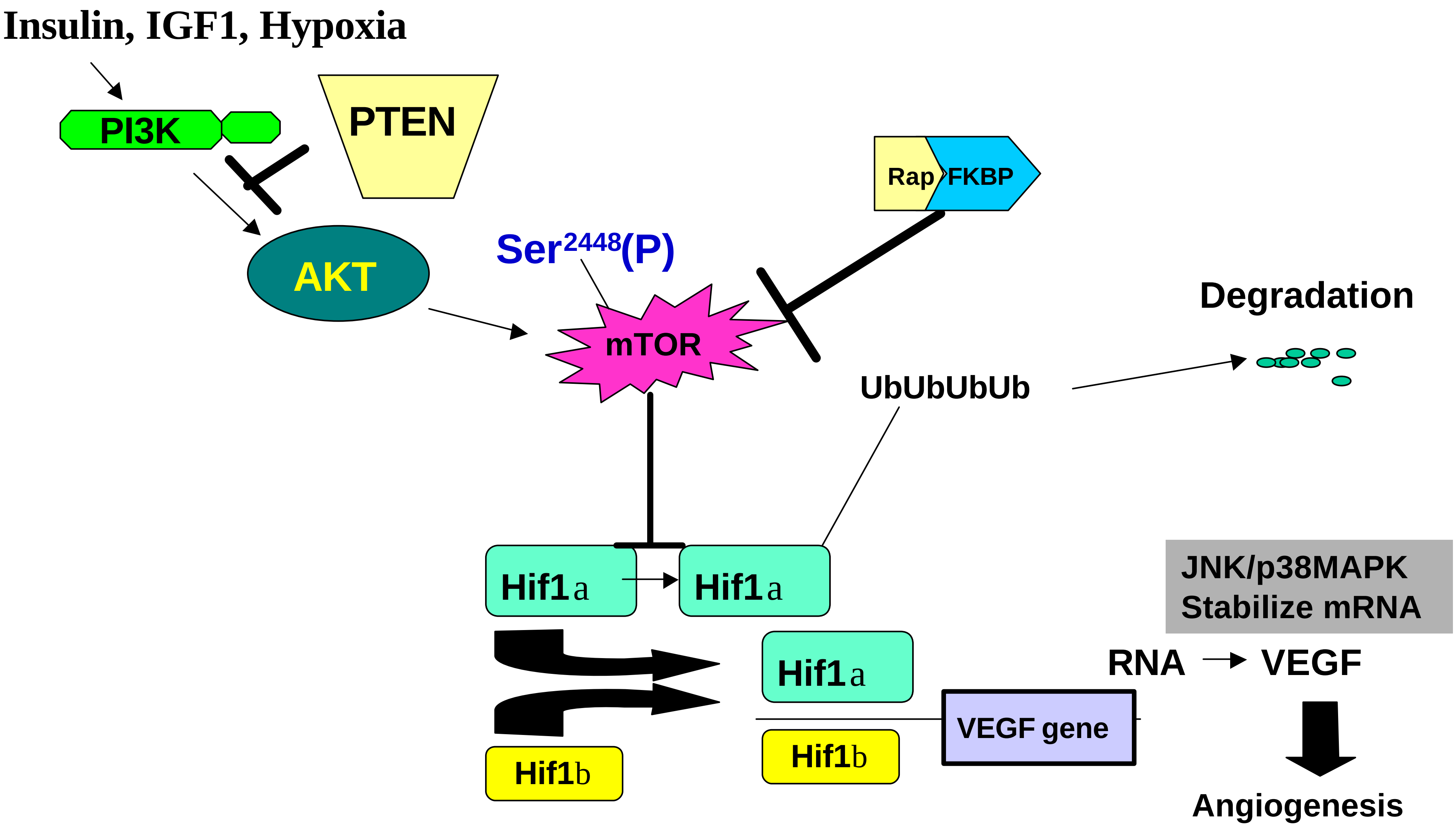
Courtesy of Dr. M. Brown

HIF-1a Protein Stability is Regulated by O_2 and by VHL Tumor Suppressor



Courtesy of M. Brown

PI3K-PTEN-AKT-mTOR signaling pathway effects on Hif1a and Angiogenesis



Courtesy of Drs. Hudson and Abraham

Objectives

- Signal Transduction Pathways

↑Hypoxia and angiogenesis in:

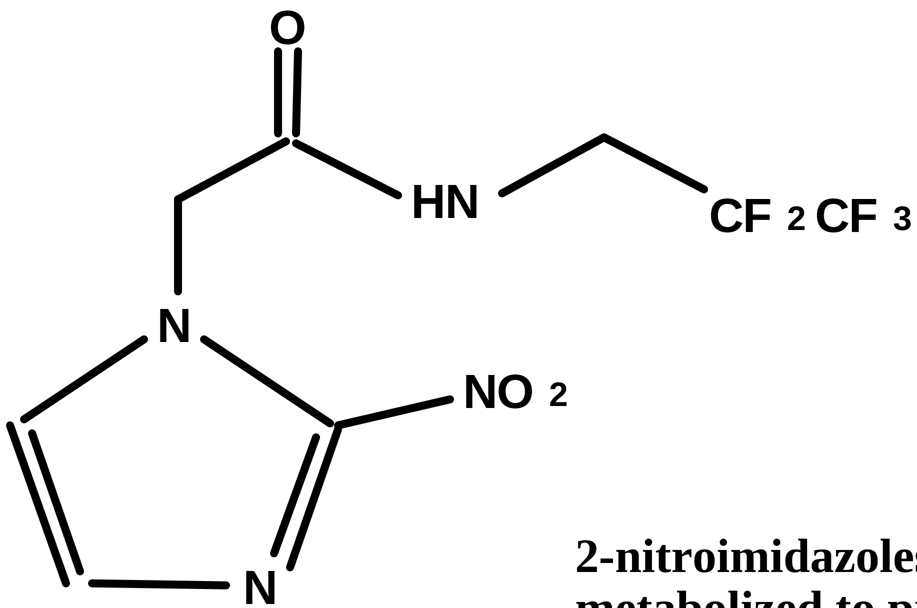
  ↑Normal tissues
  - Pathologic states
  - Tumors
- Conclusions

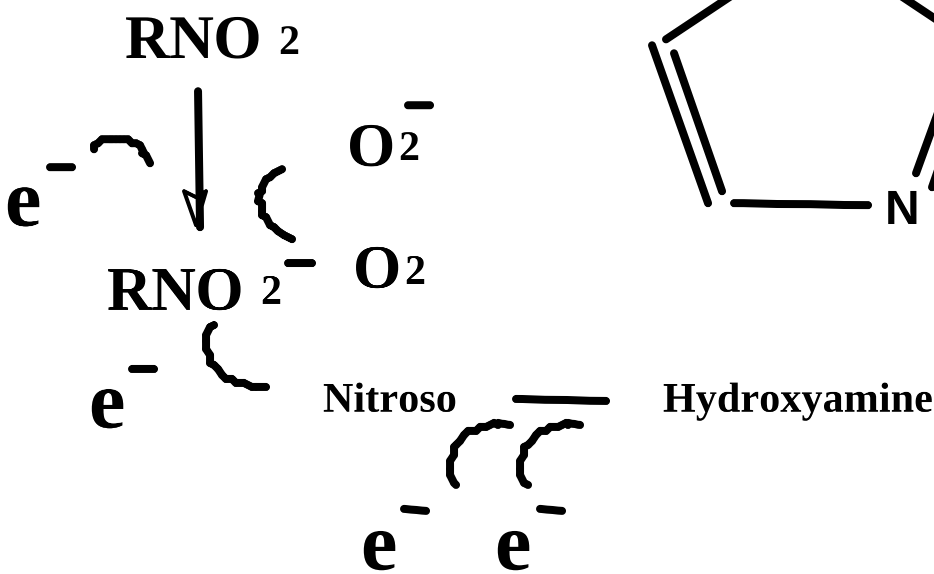
Hypoxia exists in normal tissues

- Liver
- Retina
- Thymus
- Kidney

Immunohistochemical Method to Measure Hypoxia

EF5



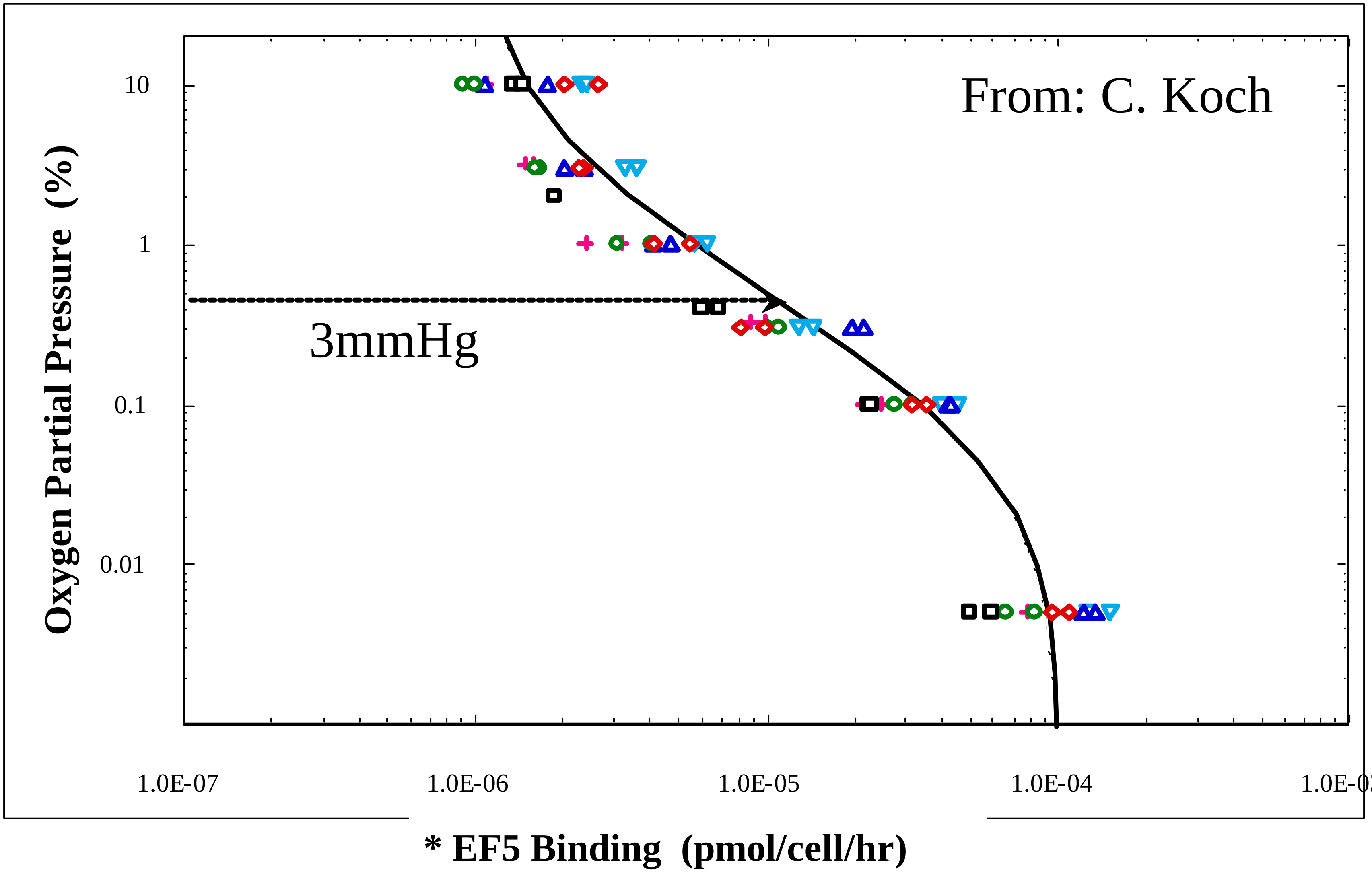


2-nitroimidazoles are metabolized to protein-thiol reactive species

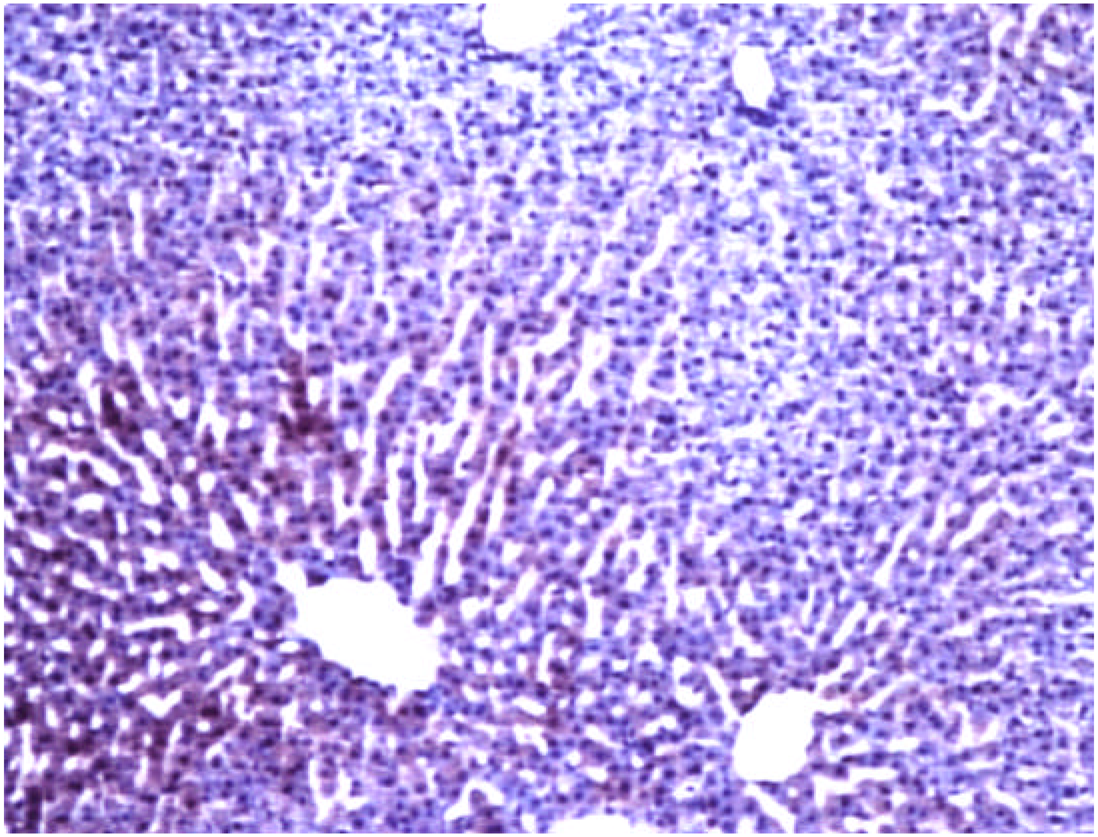
Analogue: Pimonidazole

From: Evans and Koch

Summary ^{14}C-Labeled EF5 Binding: Human Cancer Cells - w/ Biochemical Model



Peri-central hypoxia - Normal liver
Pimonidazole staining

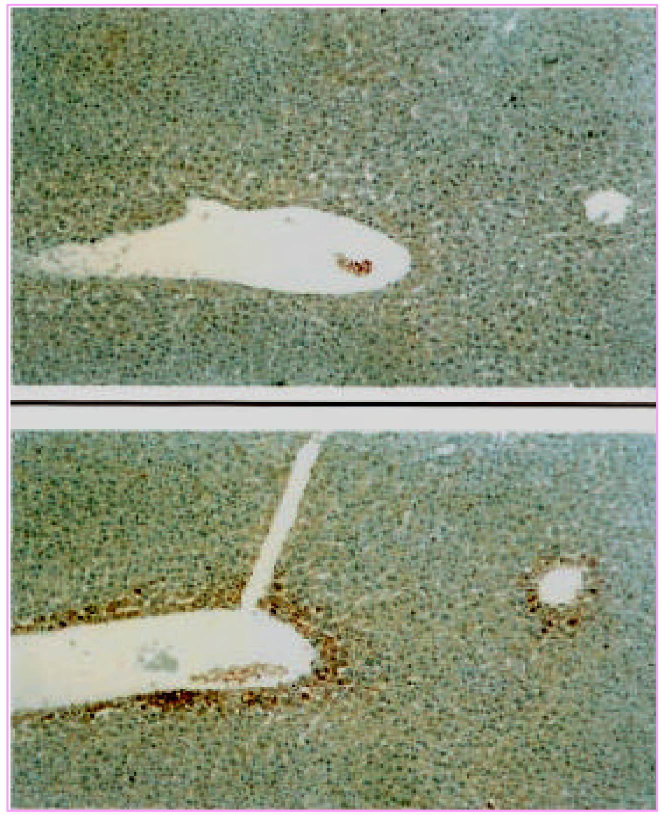


Data courtesy of: Arteel, Raleigh, Thurman

Pericentral hypoxia and VEGF colocalize in normal liver

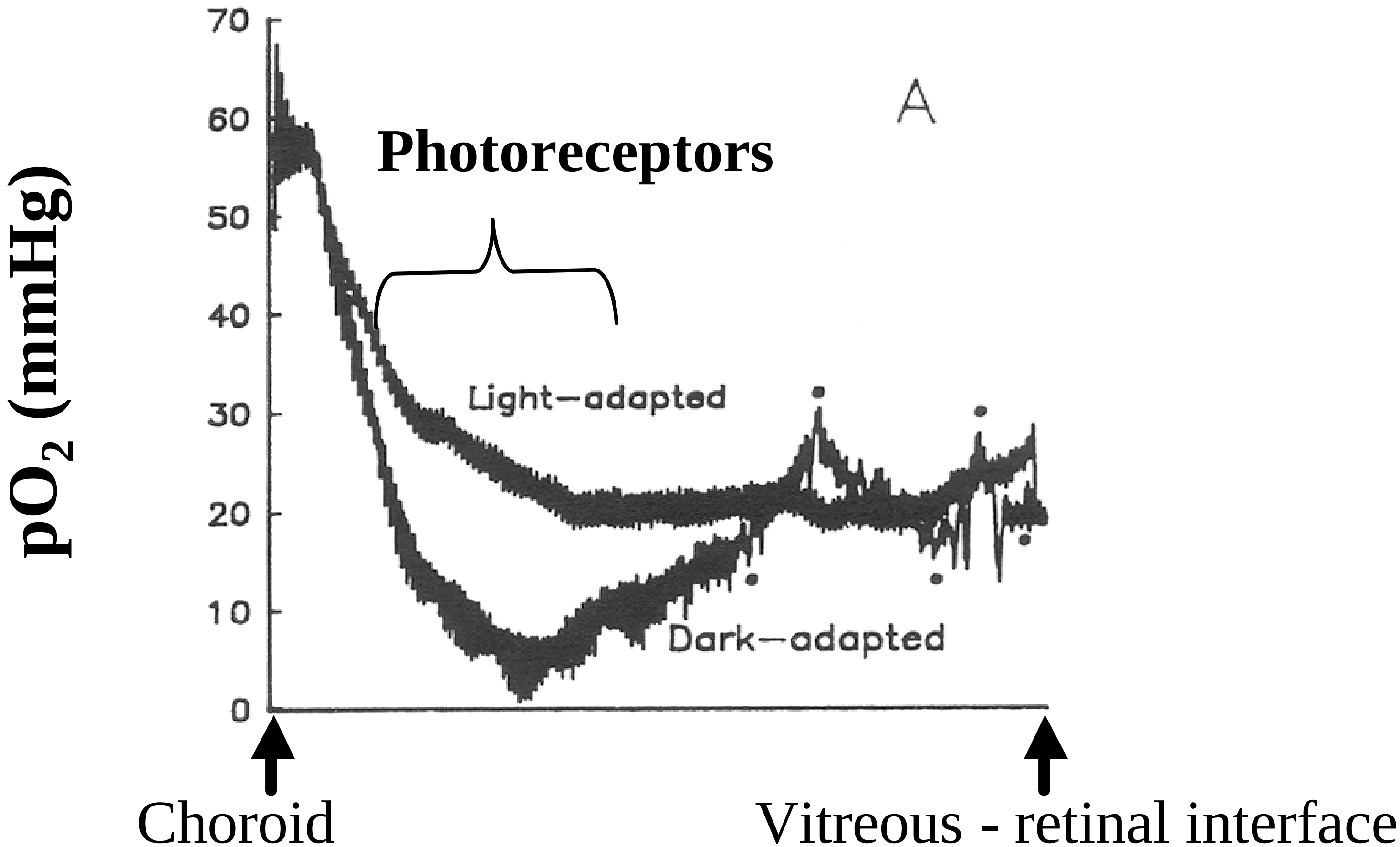
Pimo

VEGF



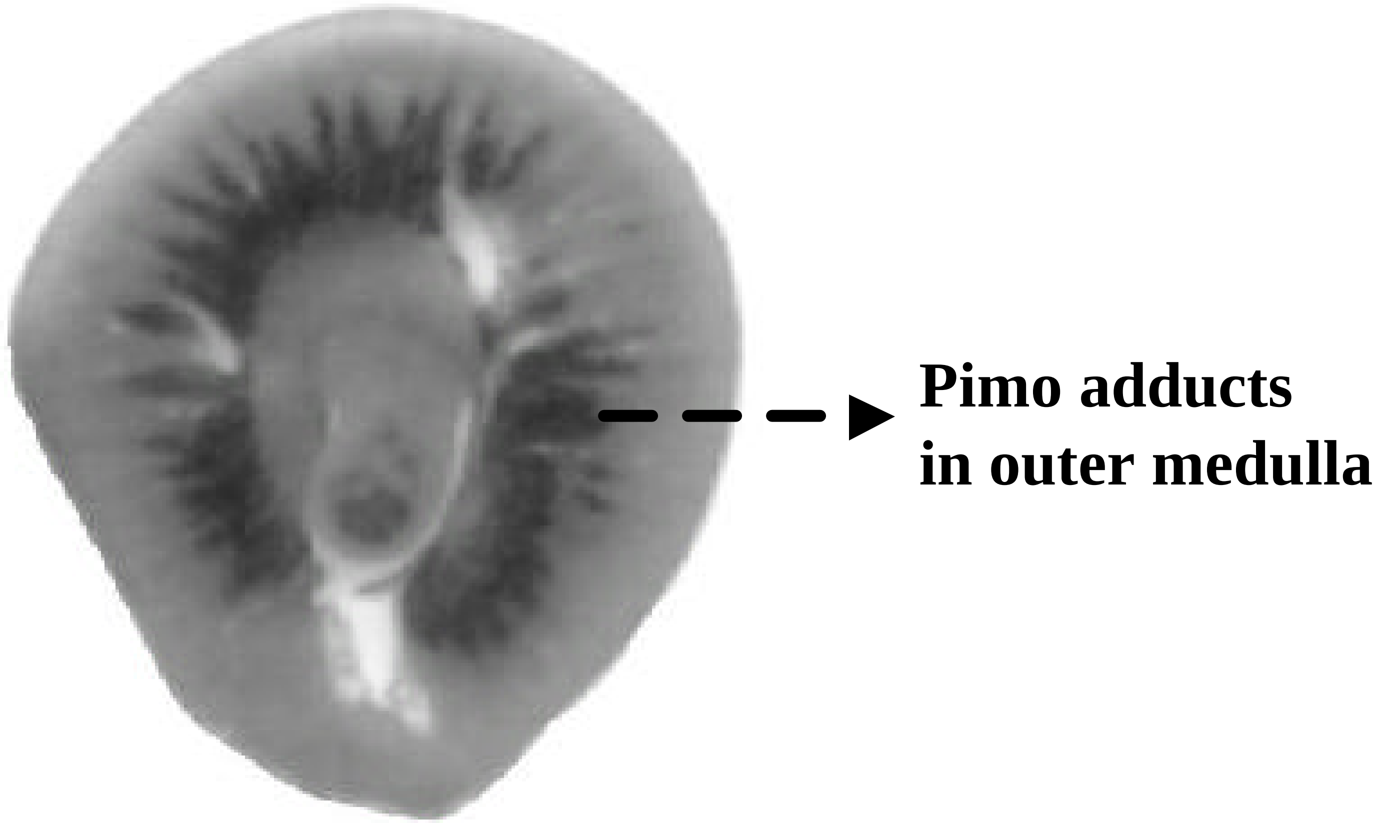
Rosmorduc *et al.*, AJP 155:1065, 1999

Retinal Oxygenation (μ electrode)
Macaque Monkey



Ahmed *et al.*, Invest Opthalmol 34:516-521, 1993

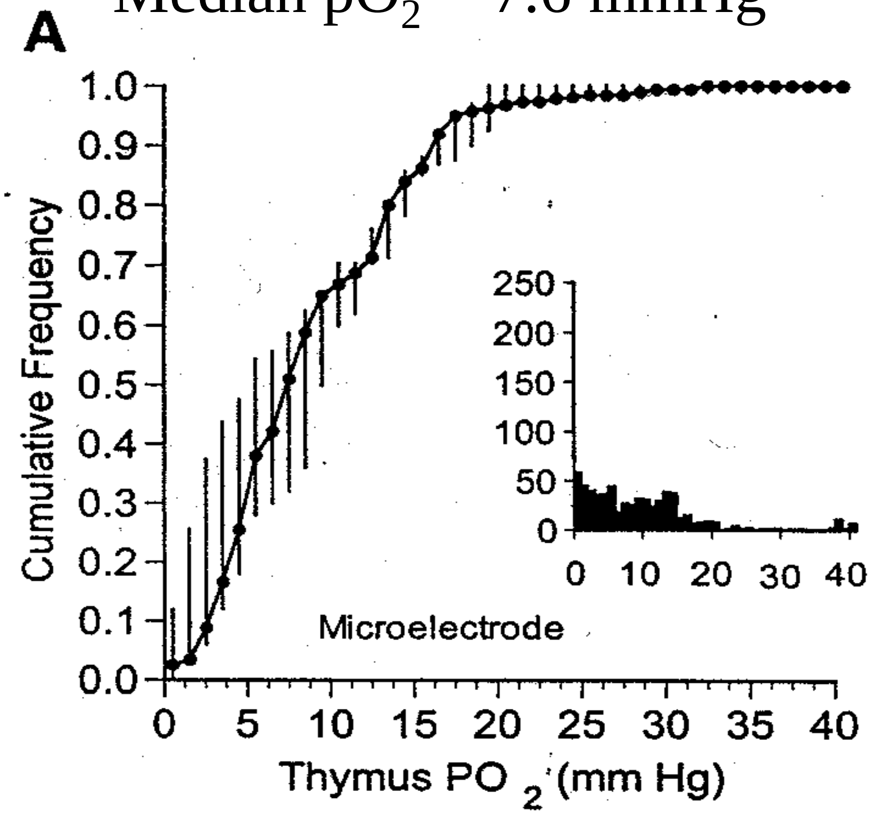
Pimonidazole uptake in renal medulla of normal rats



Data from Zhong *et al.*,AJP, 1988

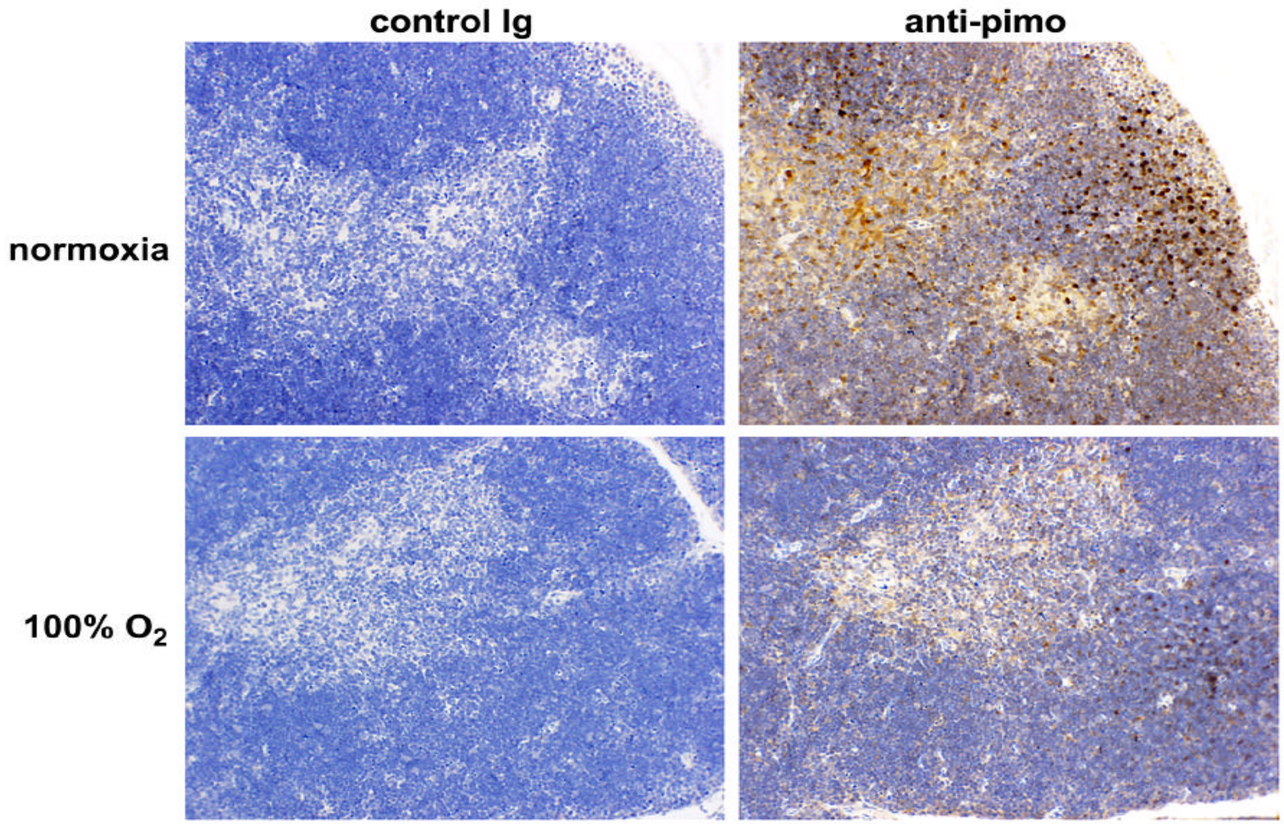
Hypoxia in Mouse Thymus: µ-electrode data

Median pO_2 = 7.6 mmHg



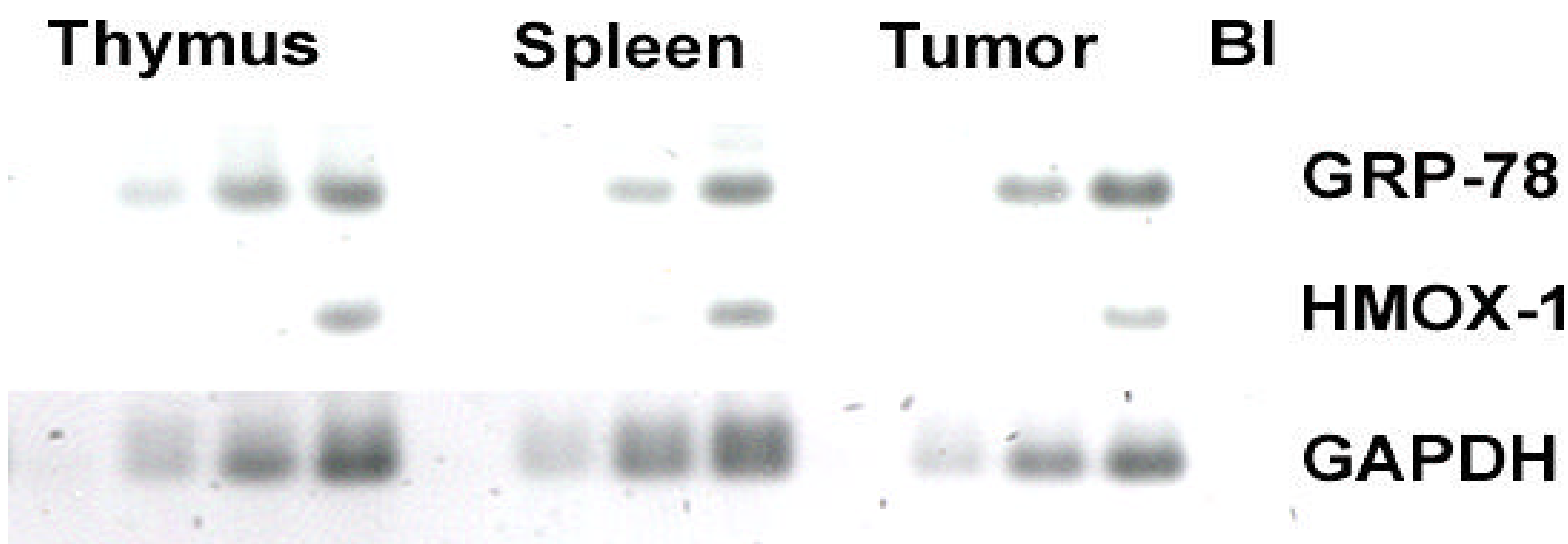
From: Braun *et al.*, AJP 2001(In press)

Pimonidazole Adducts in Normal and Hyperoxic Thymus



Data courtesy of L. Hale, DUMC 2001

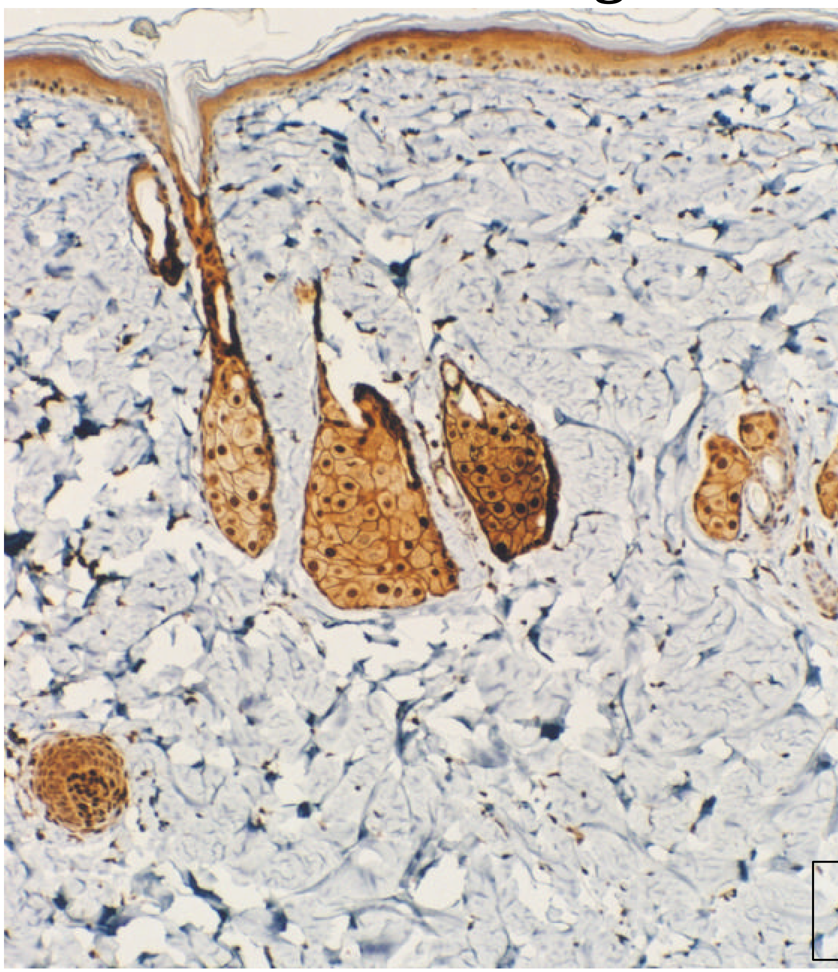
Hypoxia Responsive Genes are Expressed in Thymus



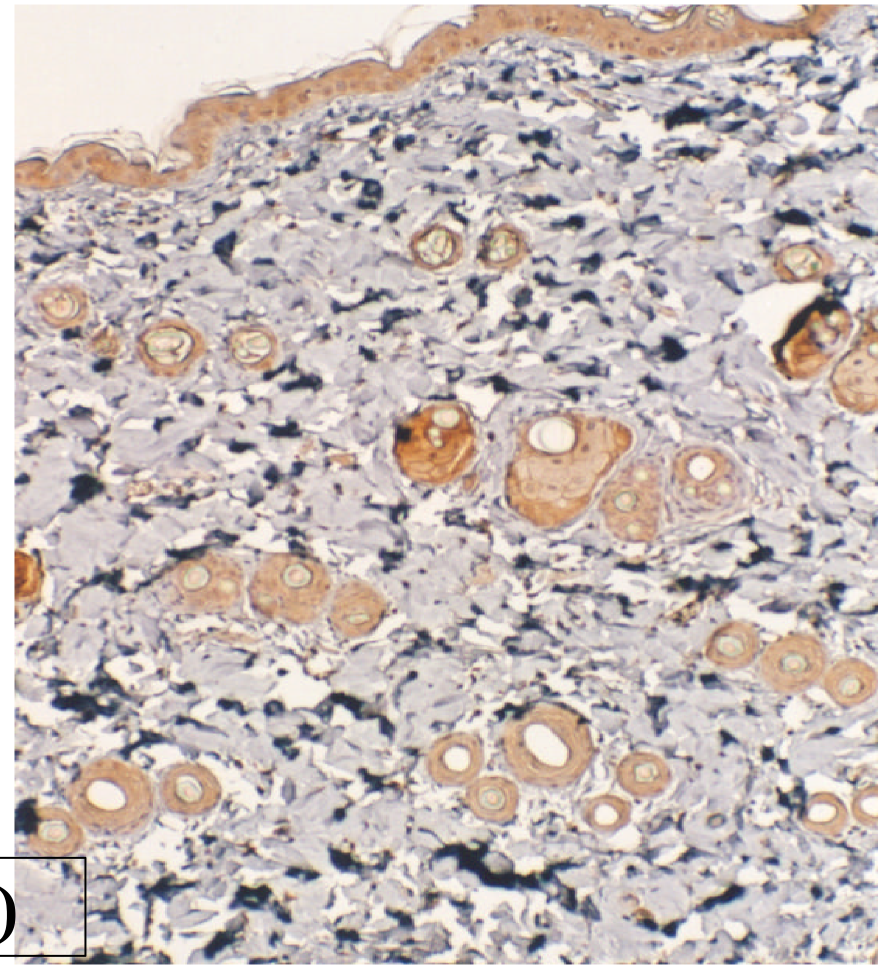
L→R: 15, 20, 25, 30 cycles RT-PCR

Hypoxia and VEGF Co-localize in Normal Rat Skin

Pimonidazole Binding



VEGF



(25X)

Haroon *et al.*, Ann Surg 231:137, 2000

Conclusion

- Naturally occurring hypoxia exists in a range of normal tissues in the presence of:
  - Hypoxia mediated gene expression
  - BUT WITHOUT INDUCTION OF Angiogenesis

Hypoxia alone may not be sufficient for angiogenesis induction

Objectives

- **Signal Transduction Pathways**

↑Hypoxia and angiogenesis in:

- **↑Normal tissues**
- **↑Pathologic states**
- **– Tumors**

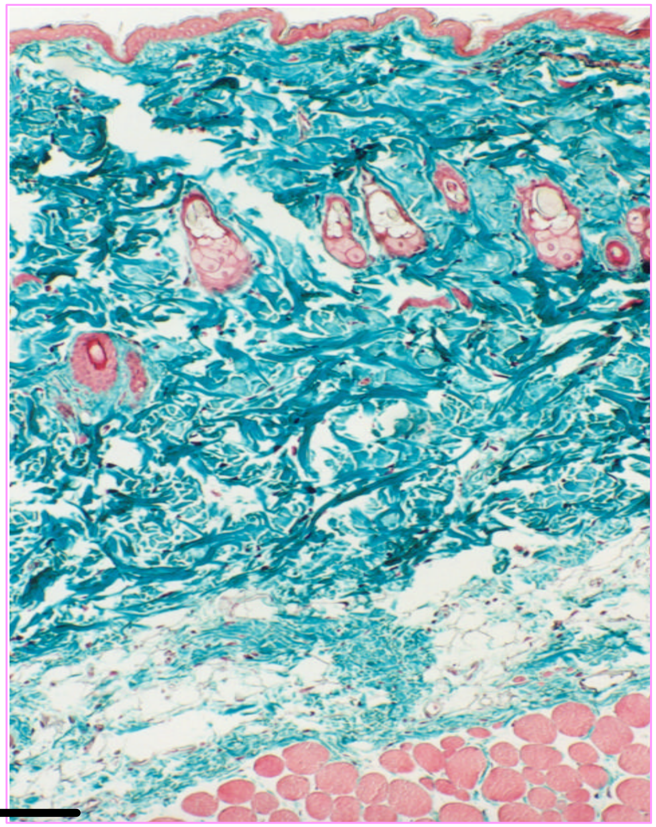
- **Conclusions**

Hypoxia / Angiogenesis in Pathologic States

- **Wound Healing**
- **Cirrhosis**
- **Diabetic Retinopathy**
- **Macular Degeneration**
- **Atherosclerosis**
- **RT induced Lung Injury**

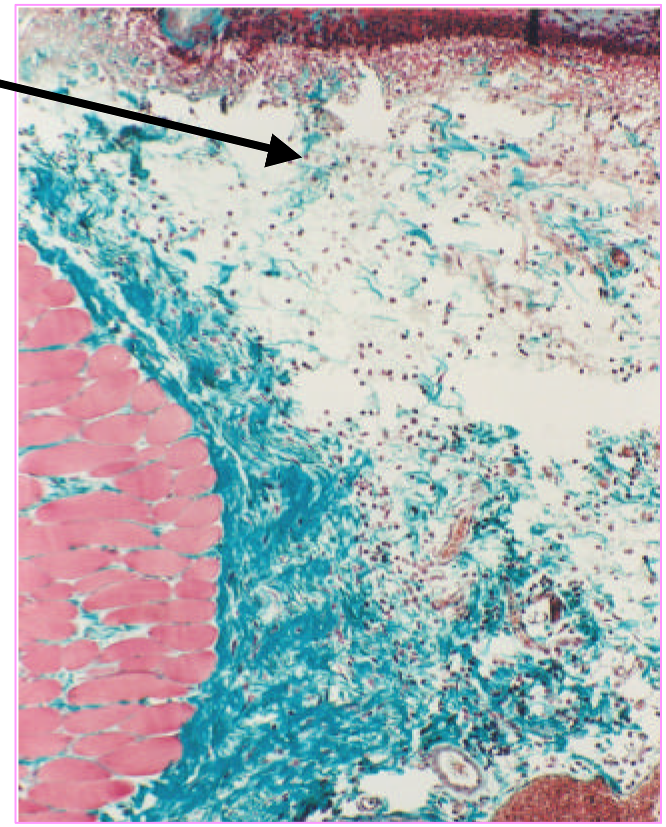
Histology of Wound Healing Reaction

Normal Rat Skin (25X)



Prov. Fibrin Matrix

Day 1 (25X)

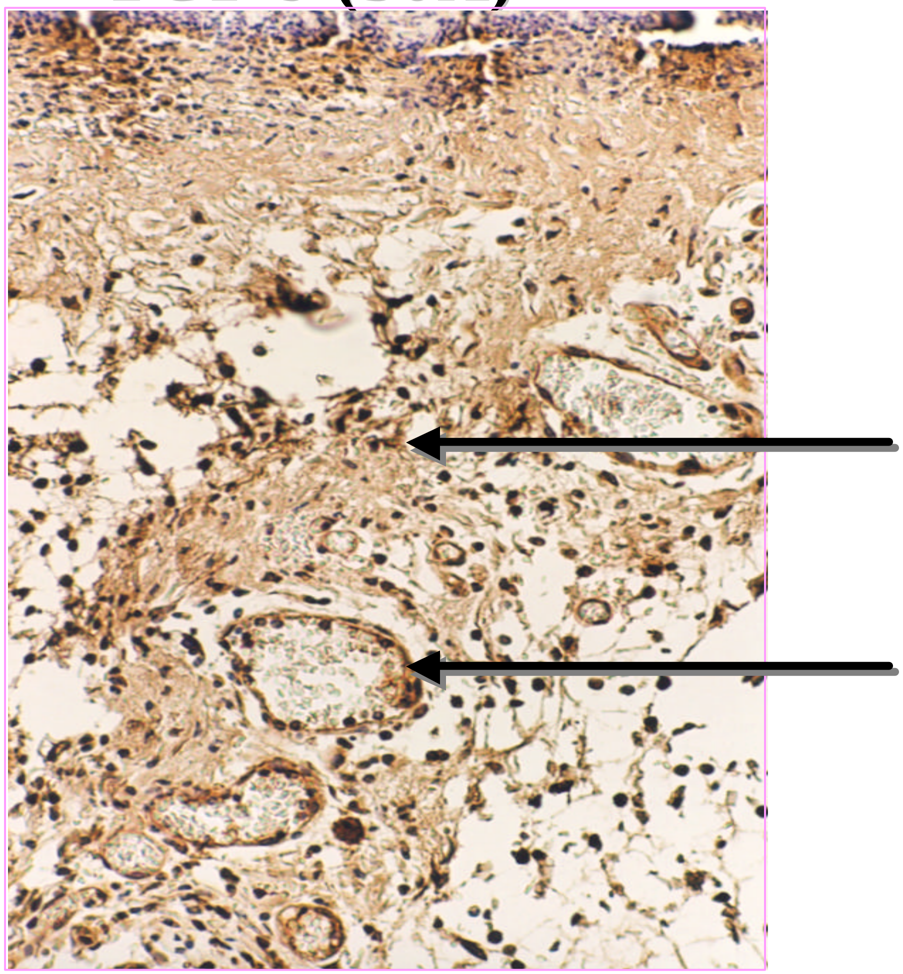


Z. Haroon, Ann Surg 231: 137, 2000

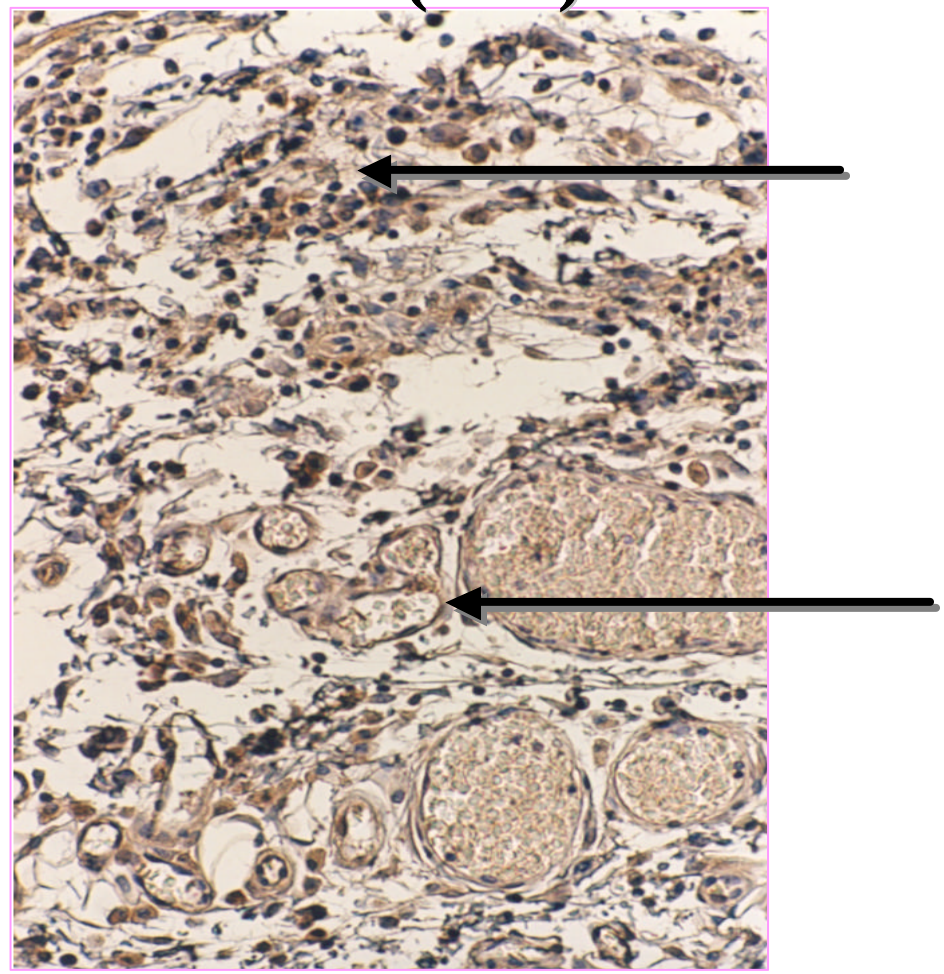
0 4 8

TGF b* and VEGF in endothelial cells & macrophages 1 day post wounding

TGF b (50X)

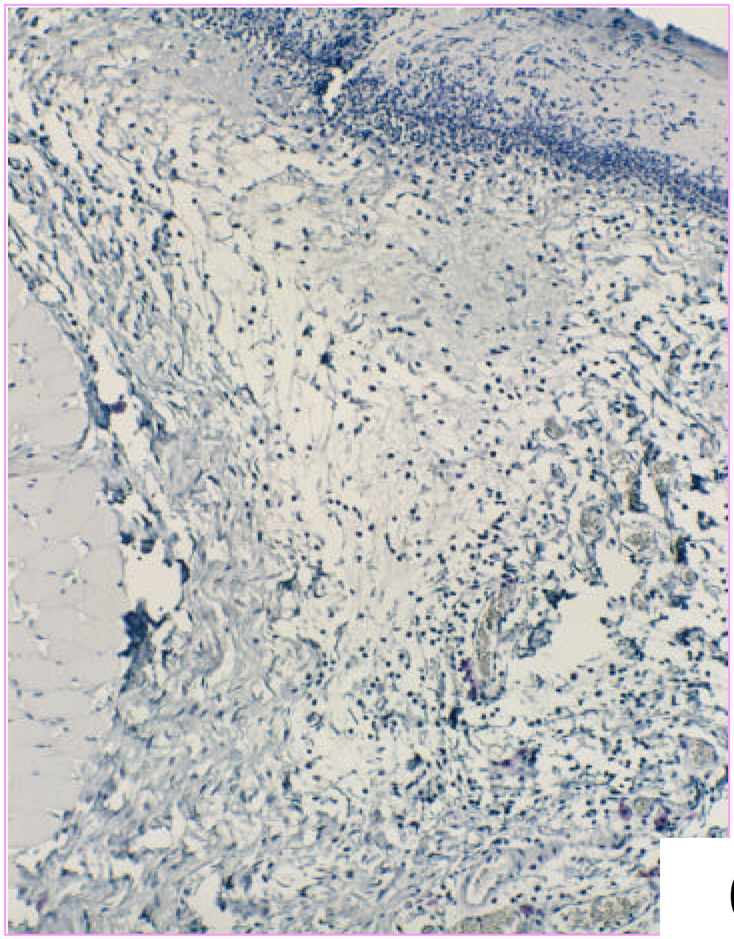


VEGF (50X)

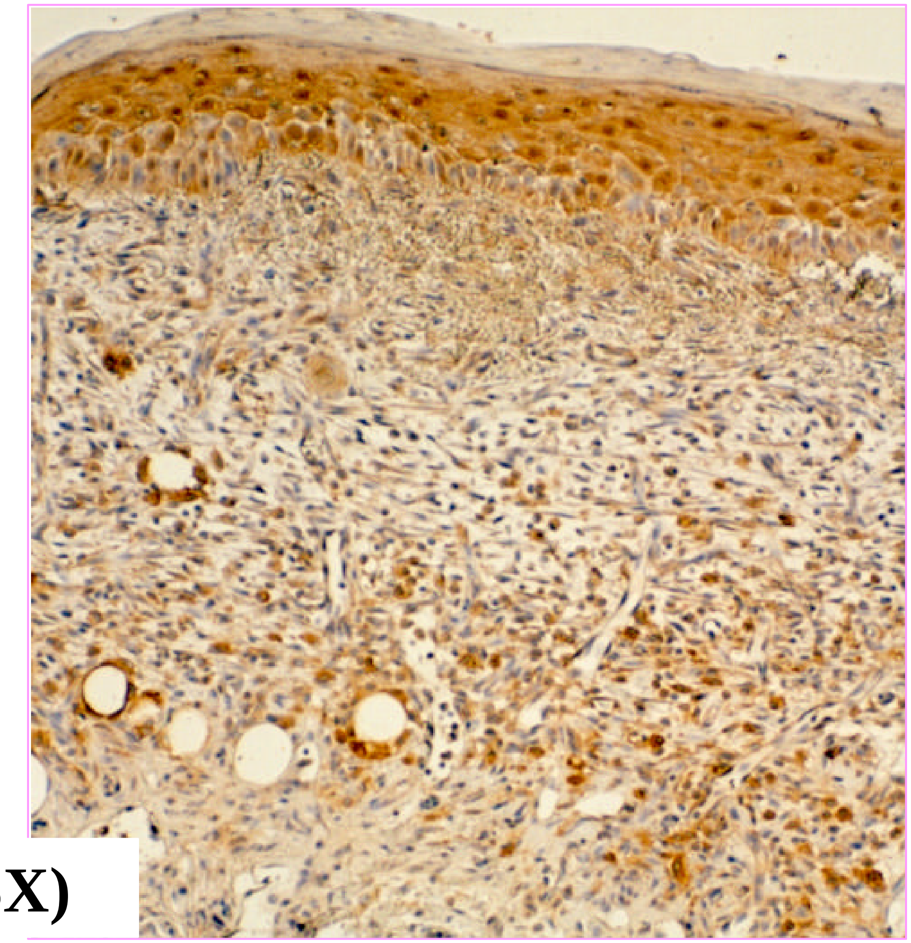


Hypoxia in Provisional Fibrin Matrix on day 1 (-) vs. at day 4 (++++)

Day 1



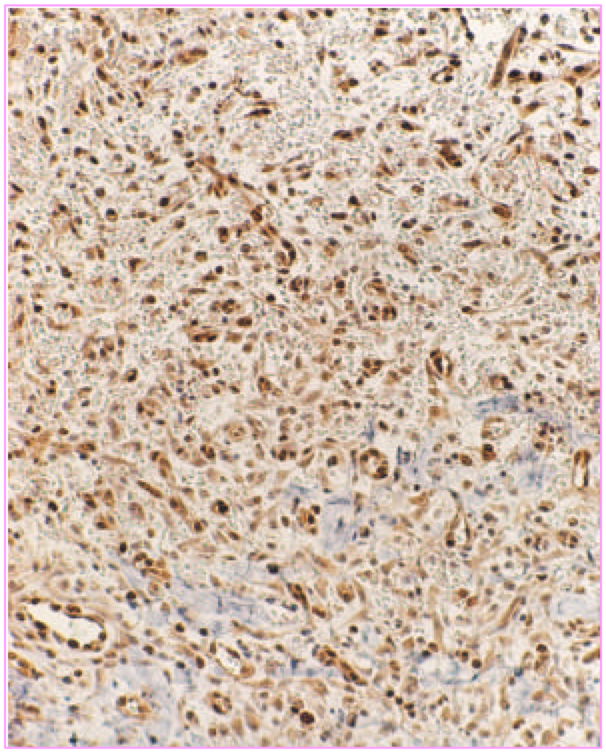
Day 4



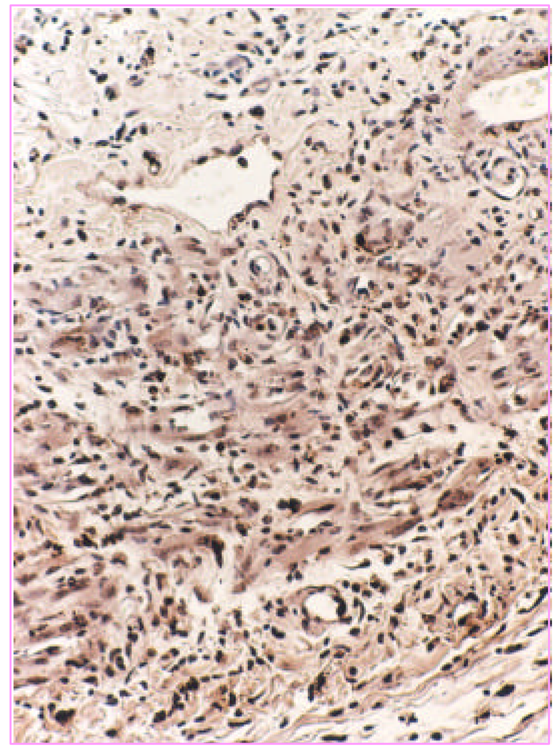
(25X)

Proliferation and Apoptosis are Maximum at Day 4

Ki67



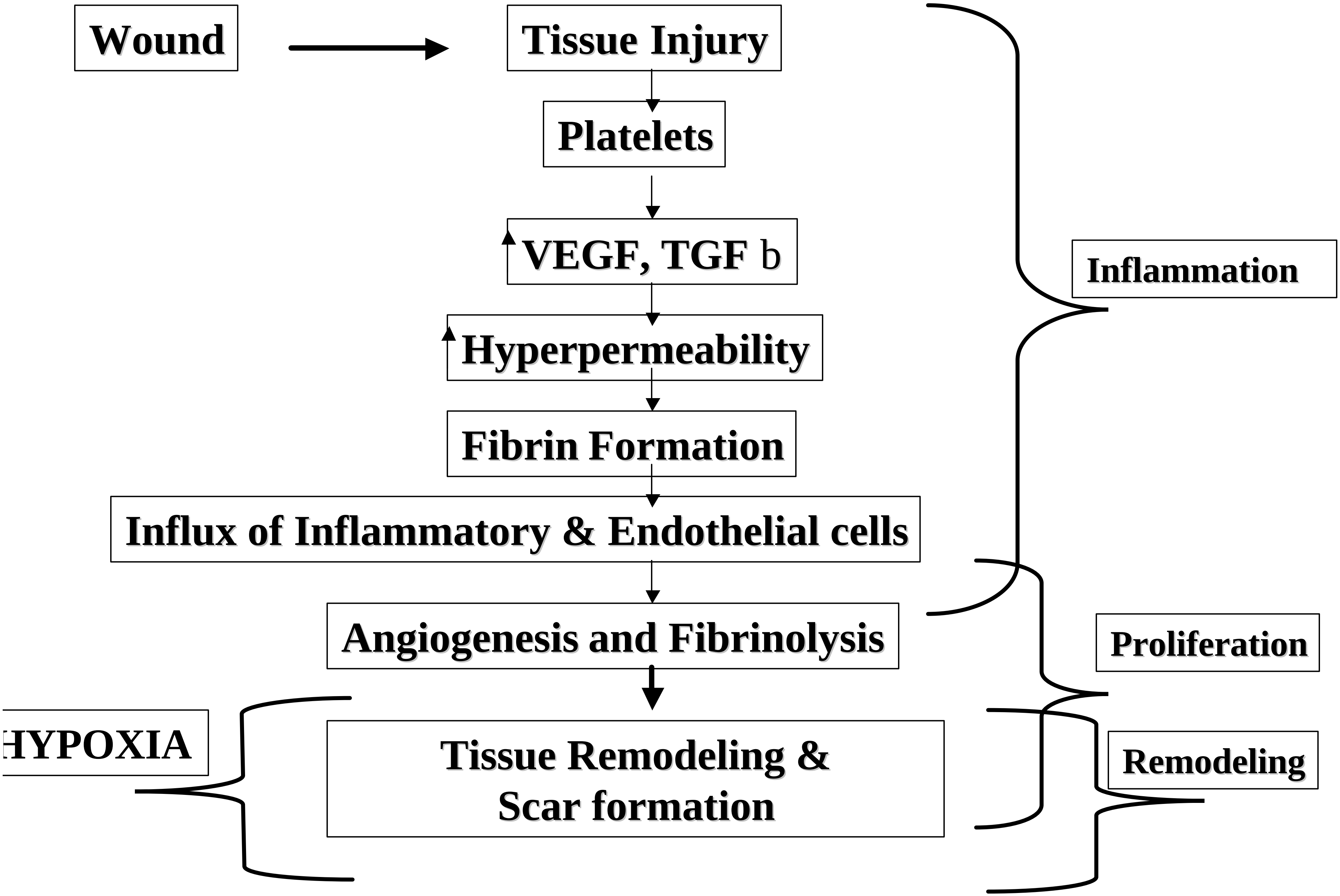
TUNEL



Hypoxic Induction of P53 / Apoptosis?

Model-wound healing

- **Angiogenesis in wound healing is initiated by**
  - **Inflammation, release of endogenous stores of VEGF**
- **Vascular remodeling / regression is associated with**
  - **High proliferation rates (O_2 Consumption)**
  - **Hypoxia**

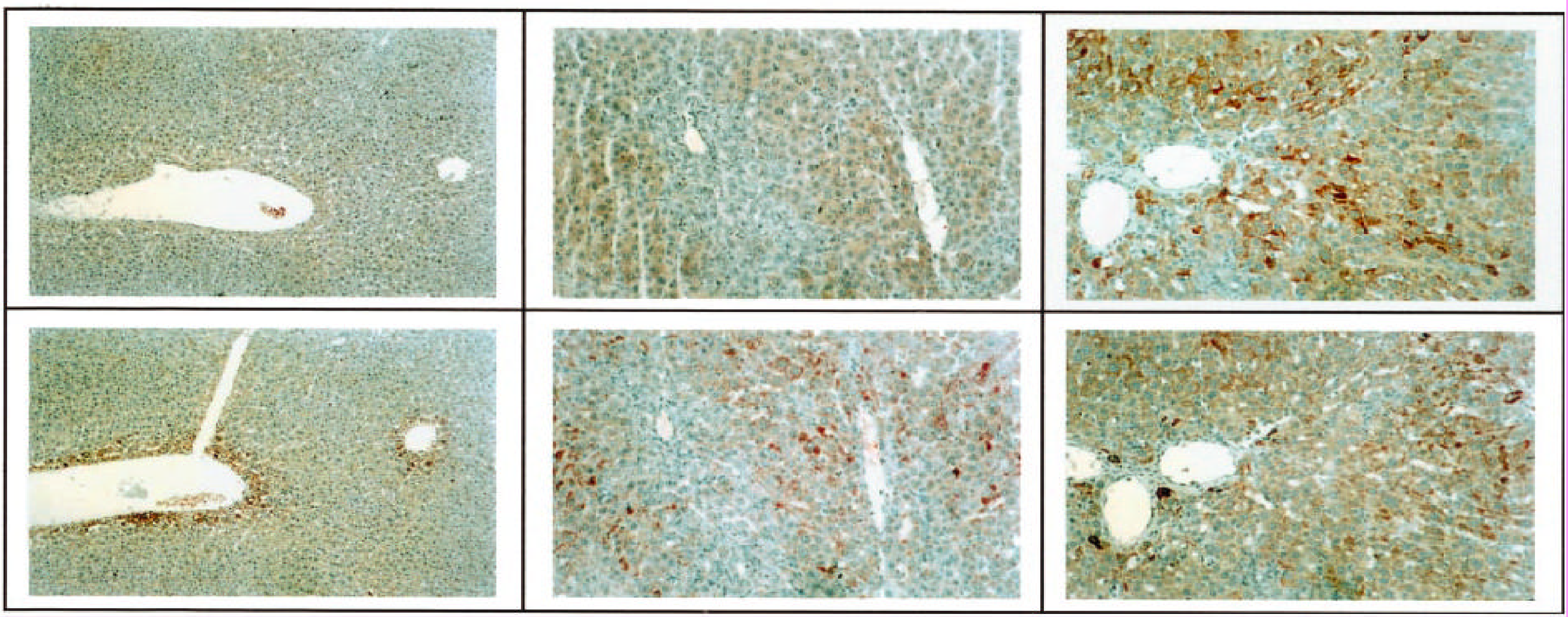

Wound
Tissue Injury
Platelets
VEGF, TGF b
Hyperpermeability
Fibrin Formation
Influx of Inflammatory & Endothelial cells
Angiogenesis and Fibrinolysis
Tissue Remodeling &
Scar formation
HYPOXIA
Inflammation
Proliferation
Remodeling

Pimonidazole vs. VEGF post bile duct ligation

Control **2 weeks** **4 weeks**

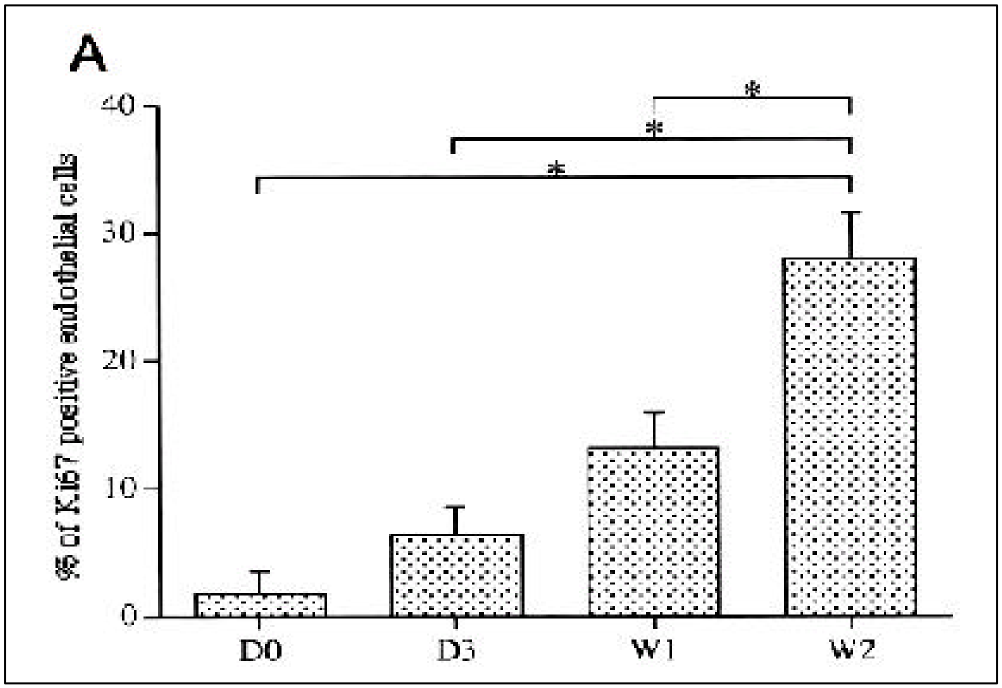
Pimo

EGF

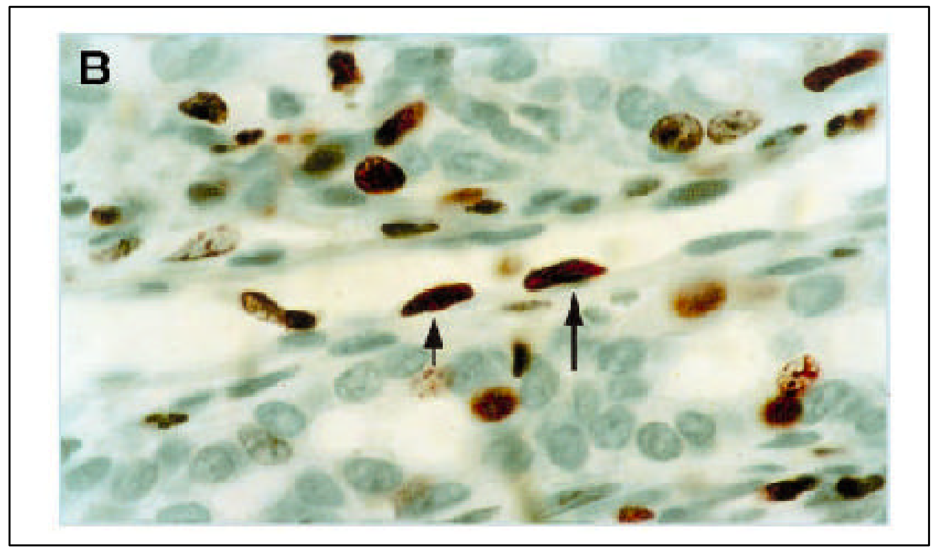


Rosmorduc *et al.*, AJP 155:1065, 1999

Angiogenesis post bile duct ligation
Early cirrhosis model



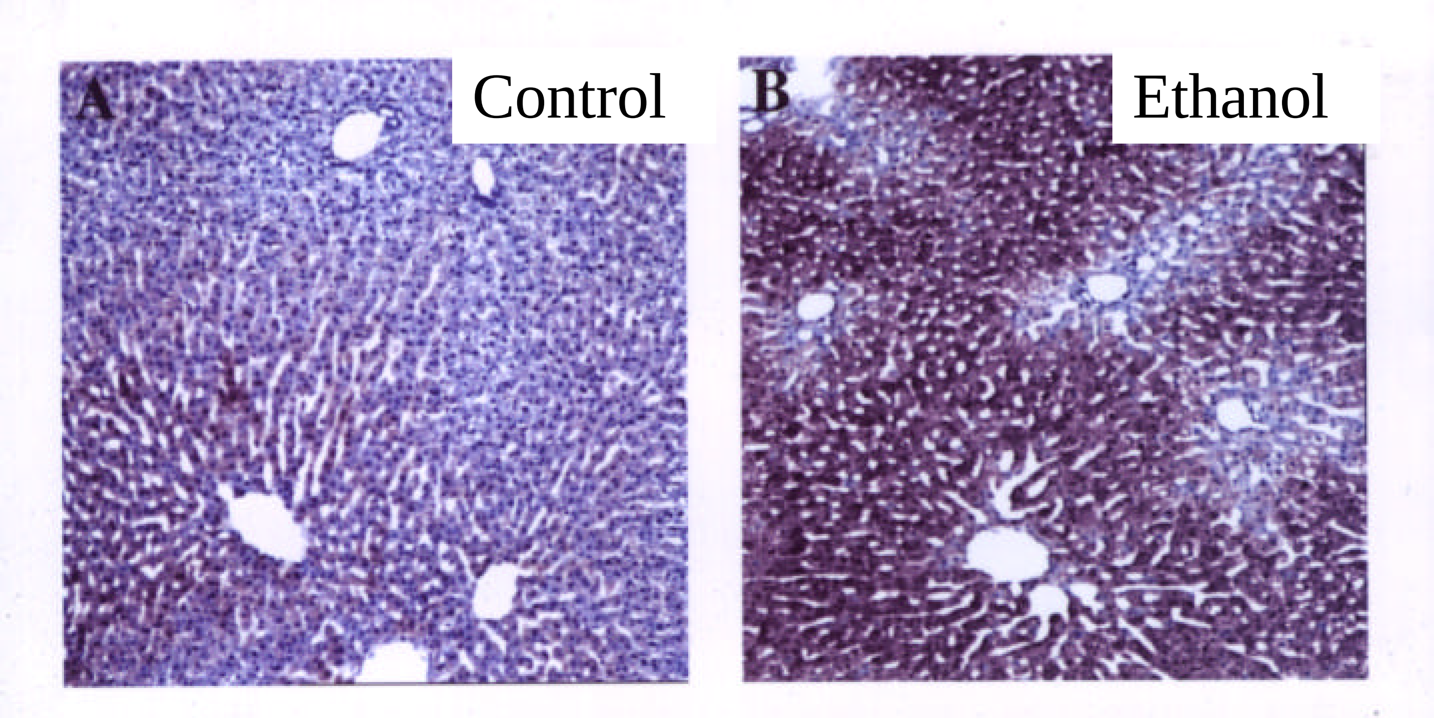
Ki67 binding



D3 = 3 days post
W1 = 1 week
W2 = 2 weeks

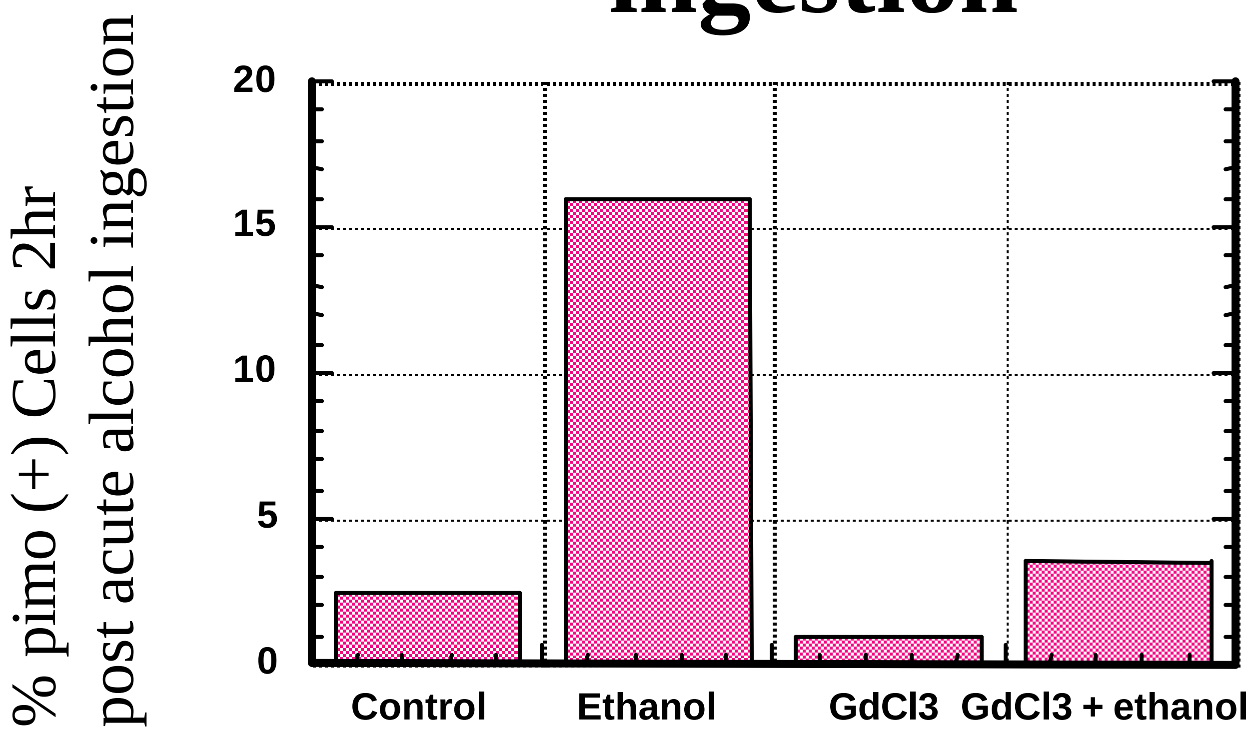
Rosmorduc *et al.*, AJP 155:1065, 1999

Liver hypoxia caused by acute alcohol (2 h post ethanol administration)



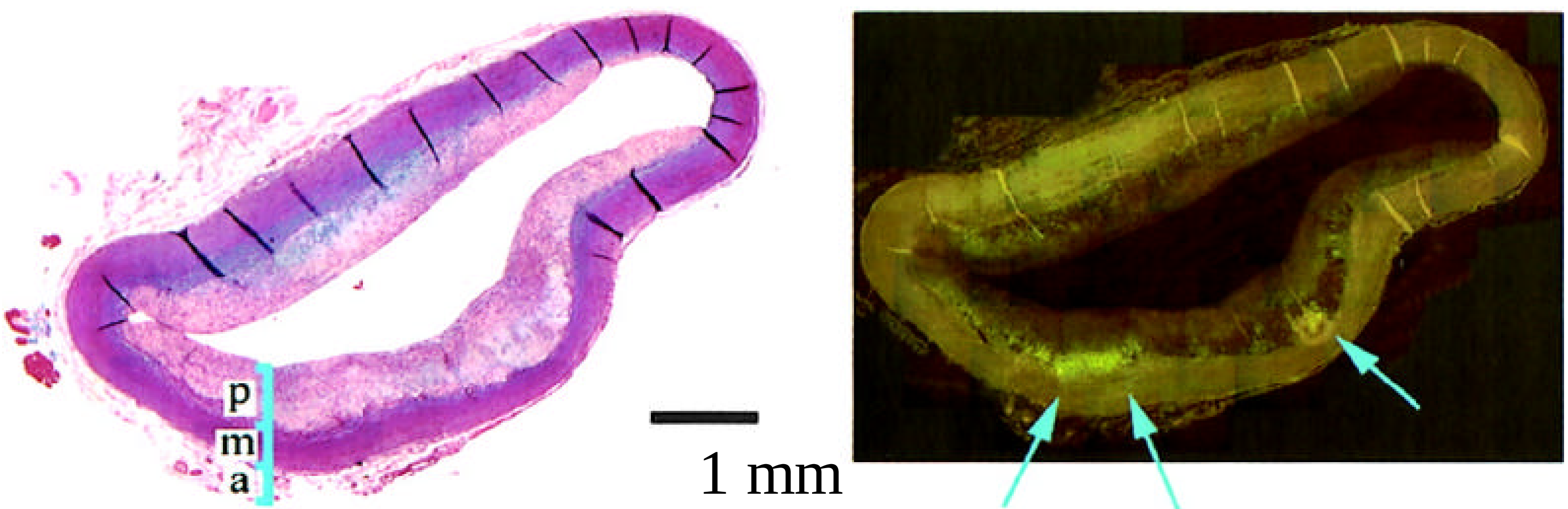
Arteel *et al.*, *Am.J.Physiol.* 271, G494-G500, 1996

Blockade of Kupffer cell activation reduces hepatic hypoxia post ethanol ingestion



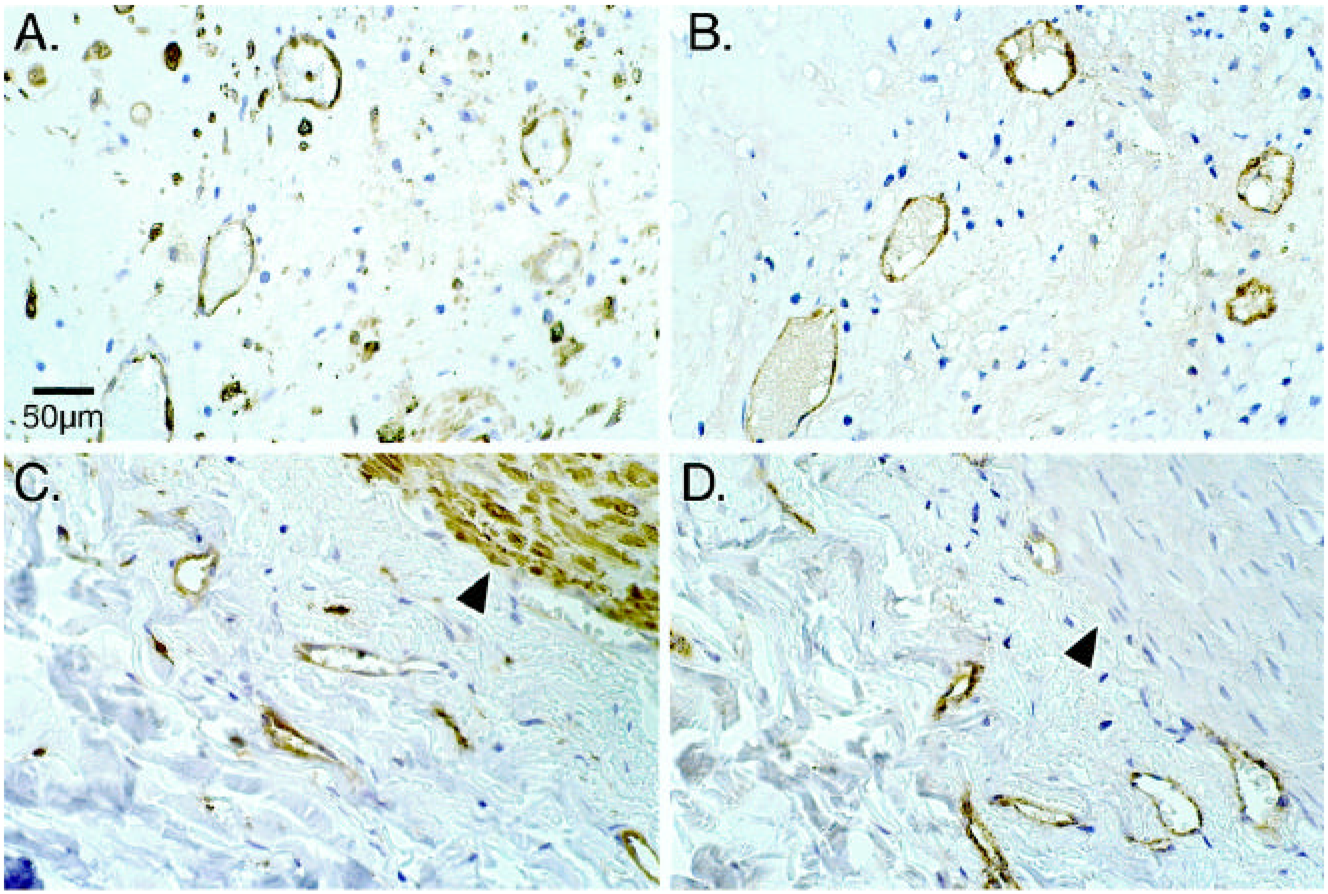
Arteel *et al.*, AJP **271, G494-G500**

NITP Binds to Atherosclerotic Plaques (rabbit model)



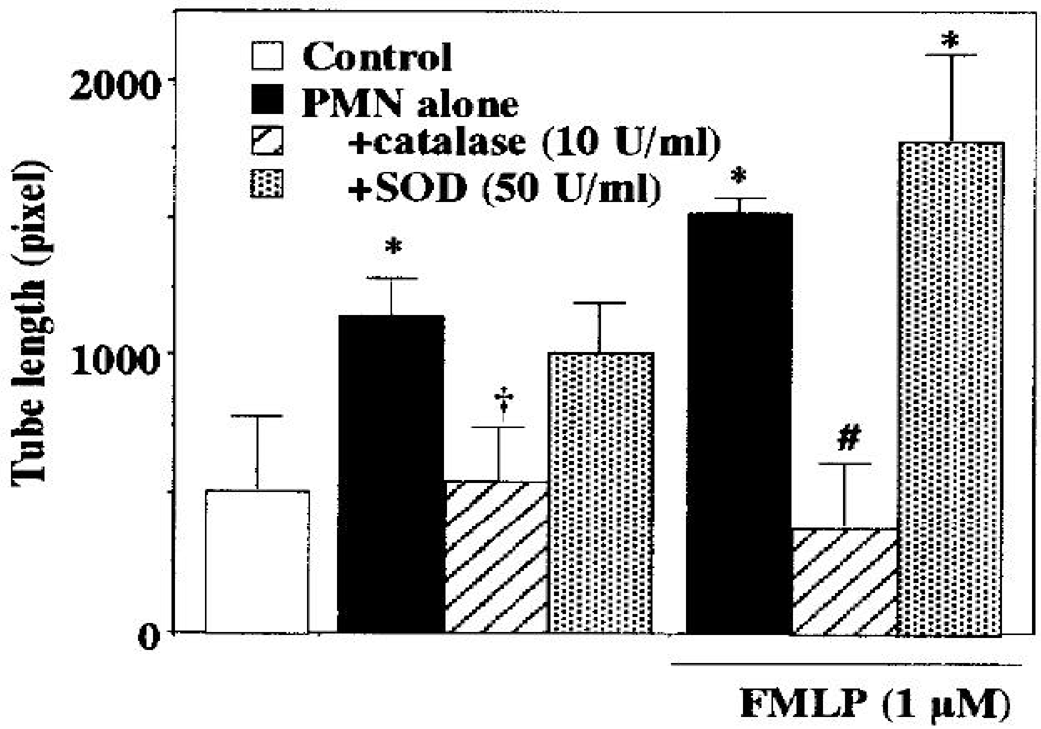
From: Bjornheden *et al.*, Art. Thromb. Vasc. Biol. 19:870, 1999

Angiogenesis accompanies plaque hypoxia



From Haroon *et al.*, Lab Invest, 2001

Catalase deactivation of H_2O_2 inhibits endothelial cell tube formation *in vitro*



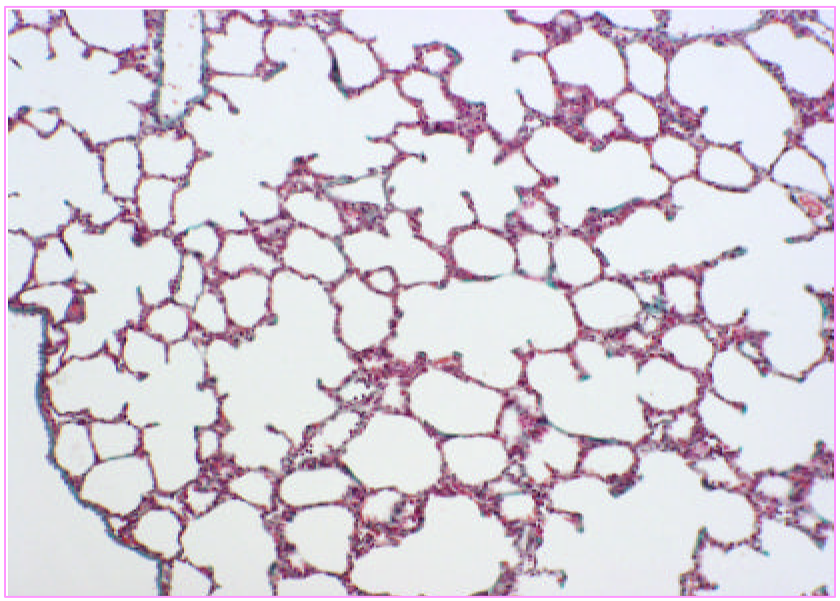
Yasuda *et al.*, Life Sci 66:2113, 2000

<u>R</u>eactive <u>O</u>xygen <u>S</u>pecies may be trigger for angiogenesis

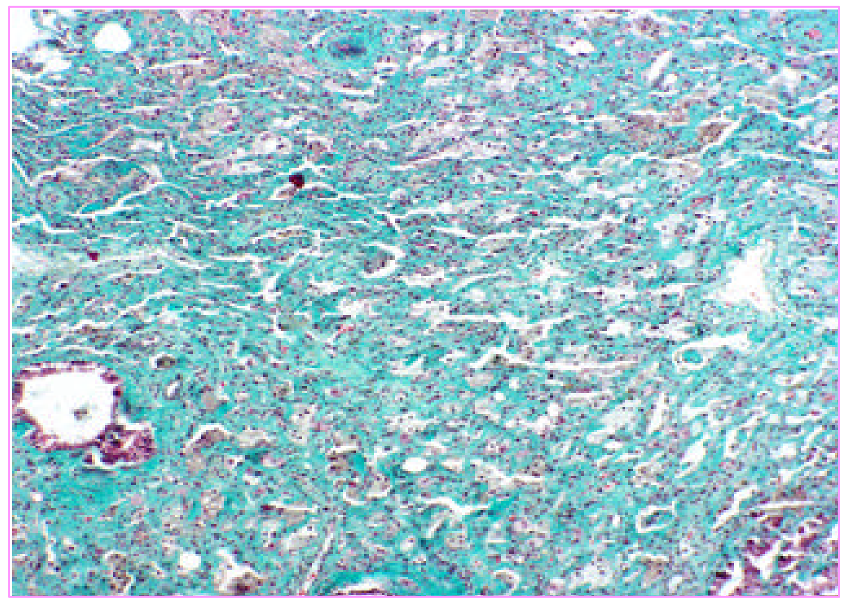
- **Lack of Angiogenesis in hypoxic normal tissues**
- **Cirrhosis model**
  - **Strong association of macrophages with hypoxia and angiogenesis**
- **Inhibition of angiogenesis with catalase**

Rat model of late radiation induced lung injury

Collagen Stain post 28Gy hemithorax



6 weeks

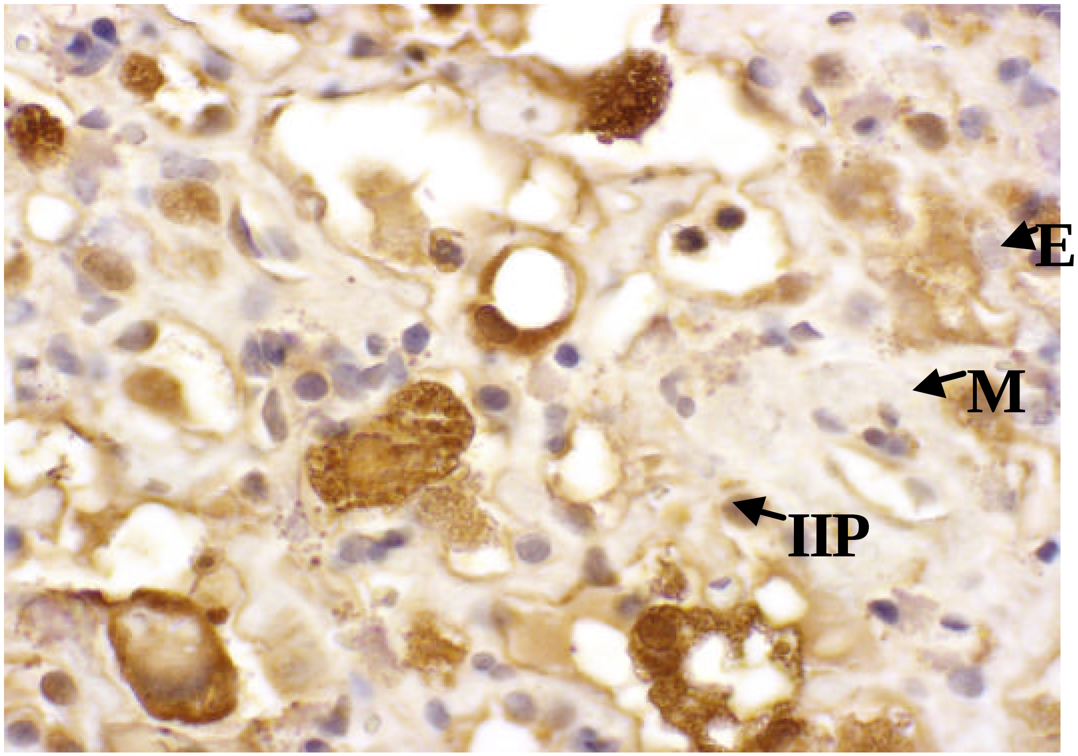


6 months

From Vujaskovic - Haroon, IJROBP, in press, 2001

Hypoxia in late radiation induced lung injury

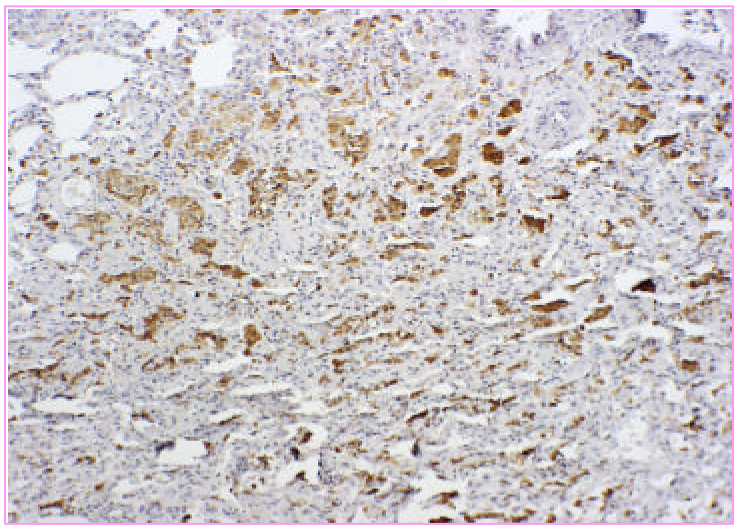
Pimonidazole uptake (hypoxia marker) @6 months



High power (40x)

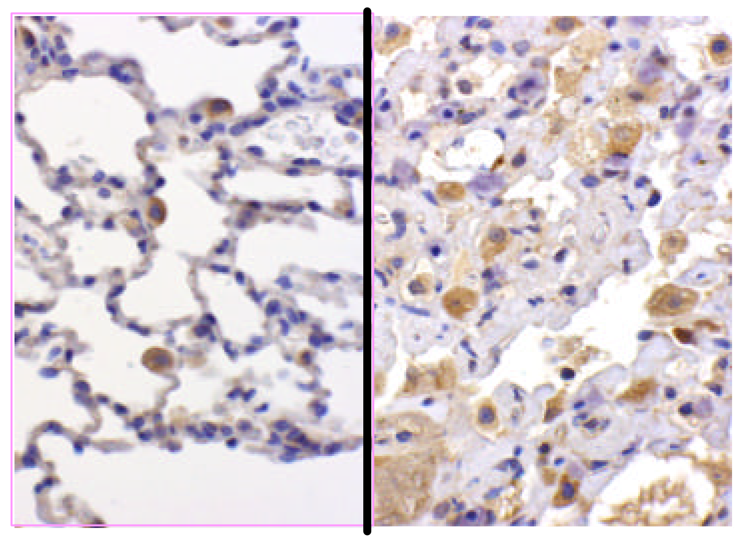
Hypoxia/ROS in late radiation induced lung injury

ED-1 (macrophage marker)



Low power (10x)

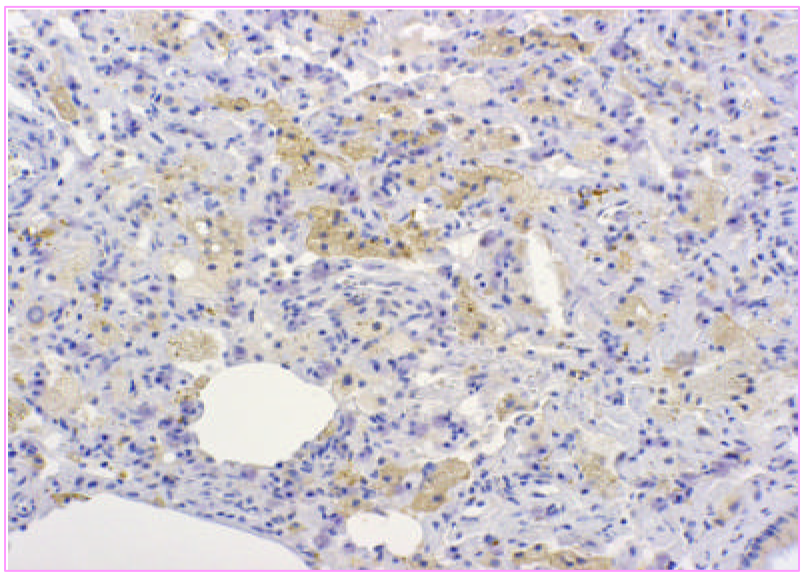
Ki67 (Proliferation marker)



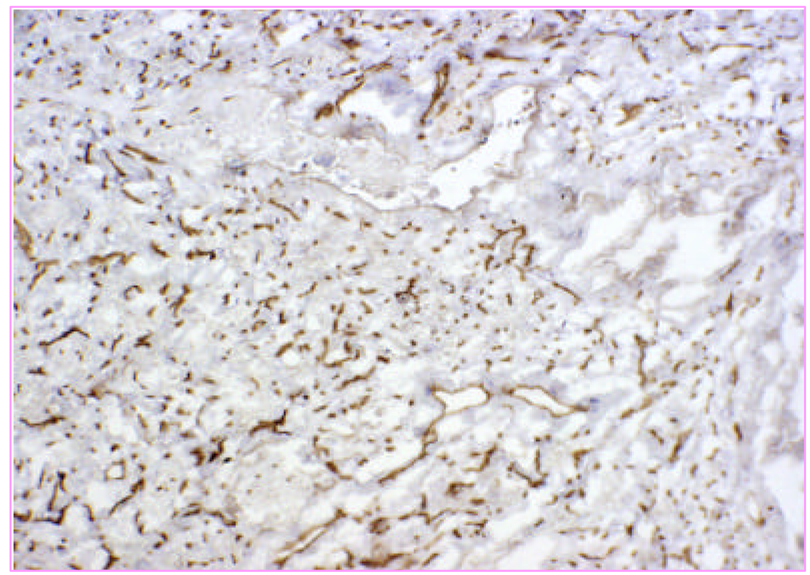
High power (40x)

VEGF and angiogenesis in late radiation induced lung injury

VEGF Expression

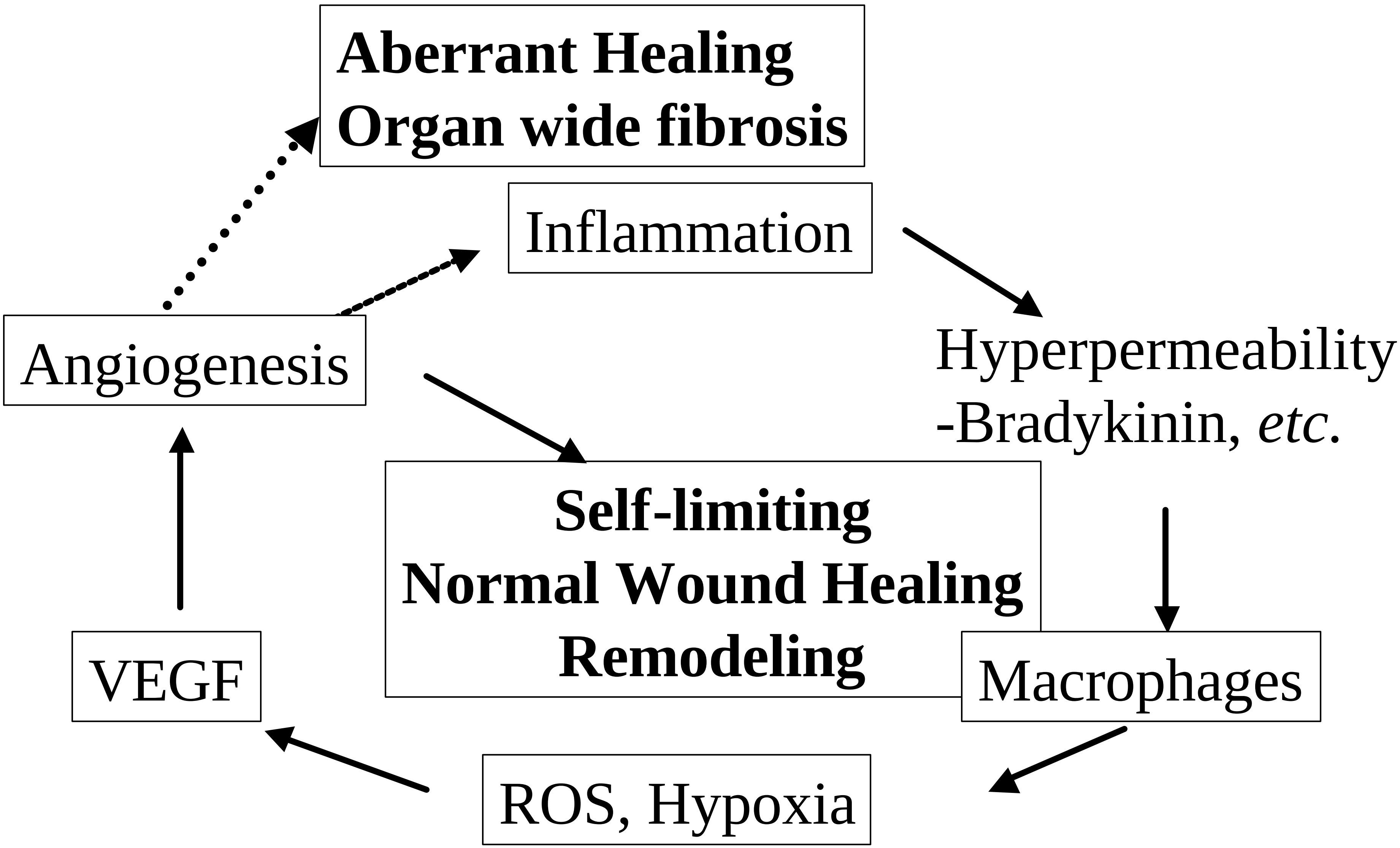


CD31 (Neovasculature)

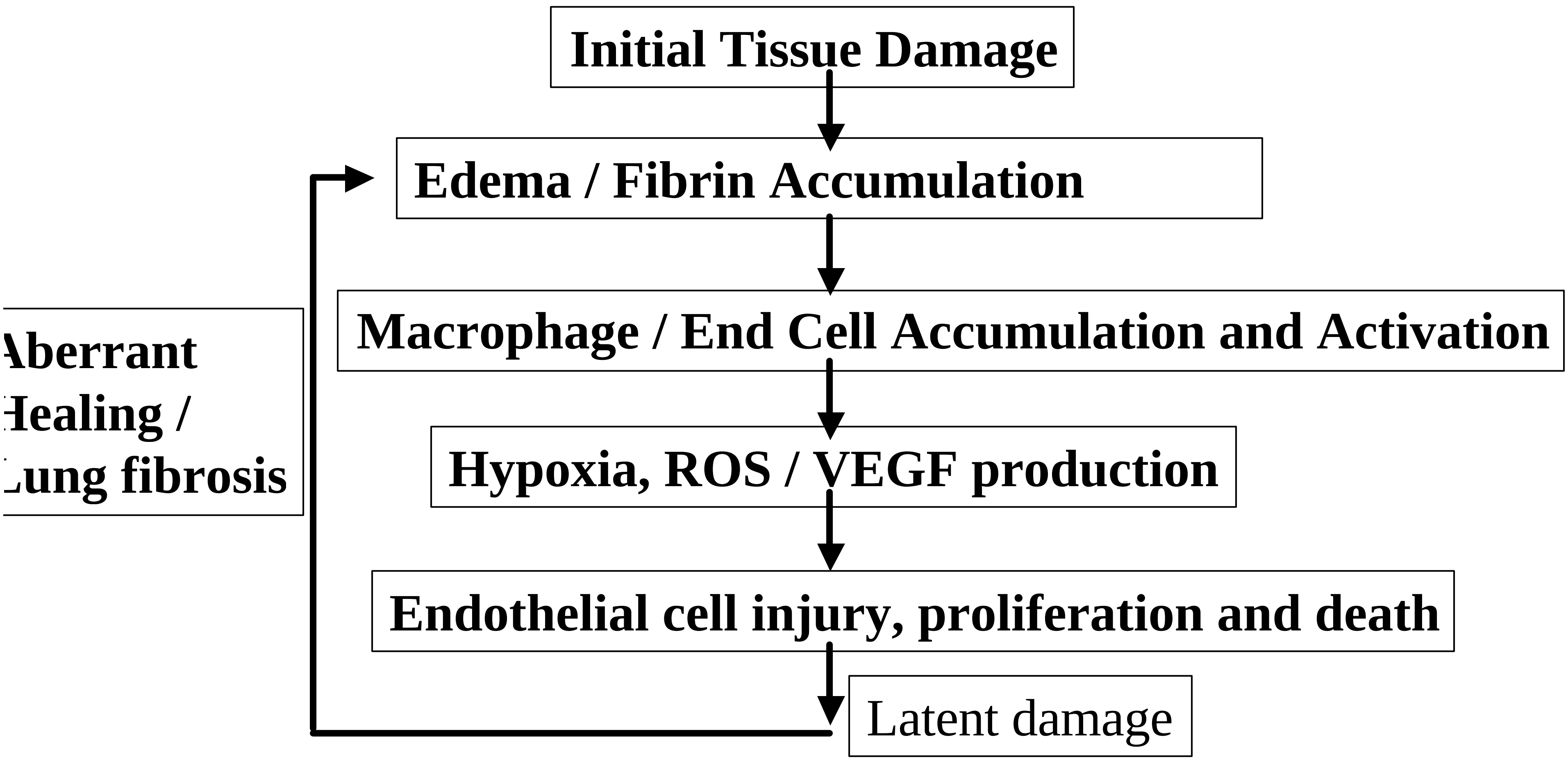


6 months post 28Gy

Conclusion - Pathologic States



Paradigm for hypoxic mediation of chronic lung injury



Objectives

- Signal Transduction Pathways
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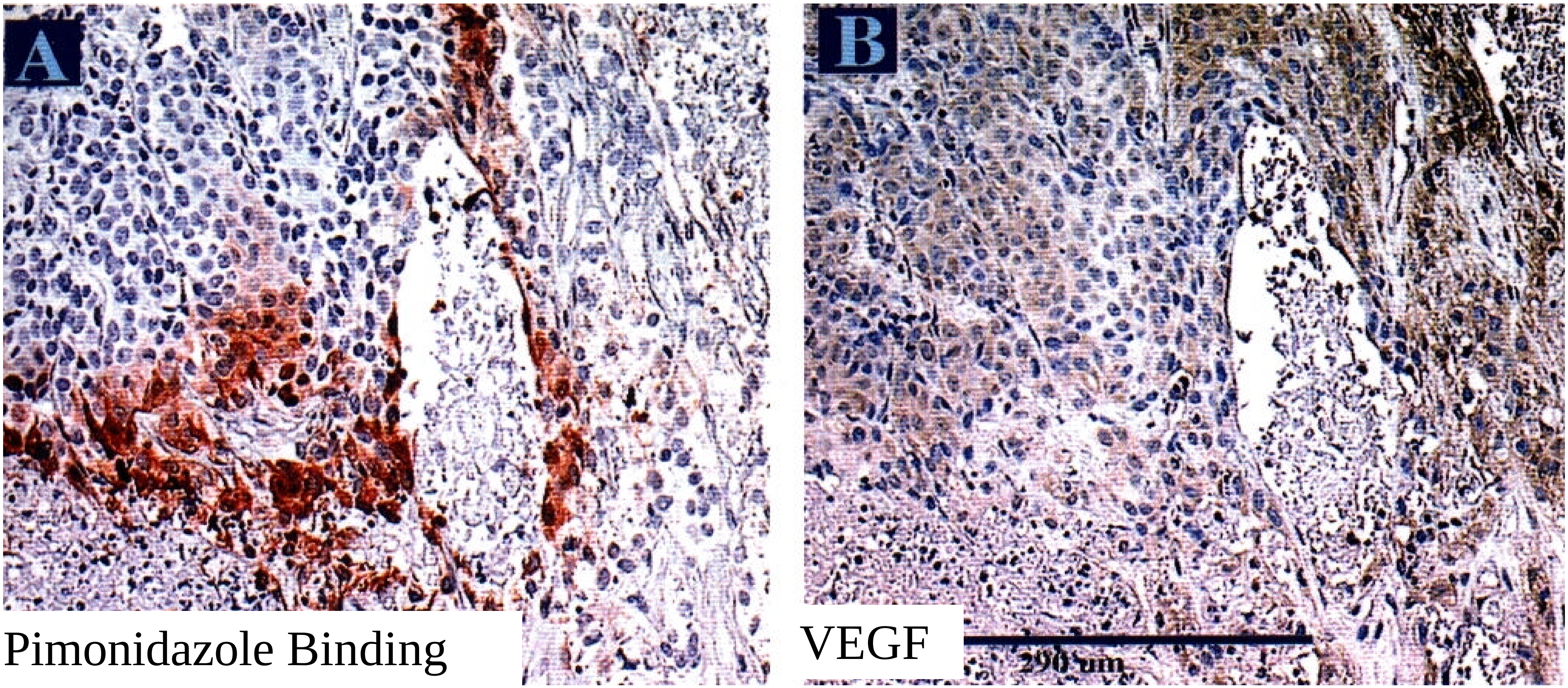
  - Tumors
- Conclusions

Questions regarding tumor angiogenesis

- What is the relation between hypoxia and angiogenesis?
- Is early angiogenesis driven by hypoxia?
- Is vascular remodeling driven by hypoxia?
- Does a tumor simulate a wound that does not heal?
  - Is there a role for the macrophage in tumor angiogenesis?

VEGF and Hypoxia - Human Cervix Cancer

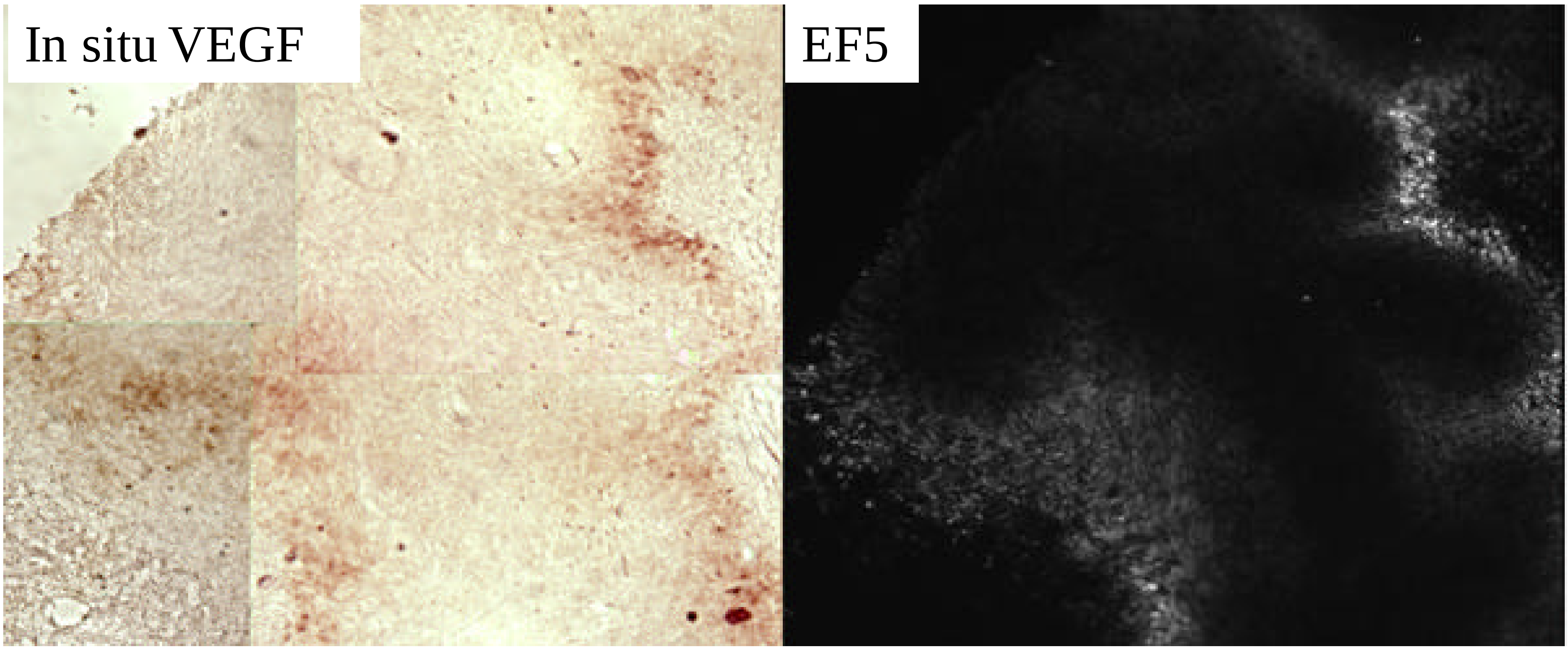


Pimonidazole Binding

VEGF

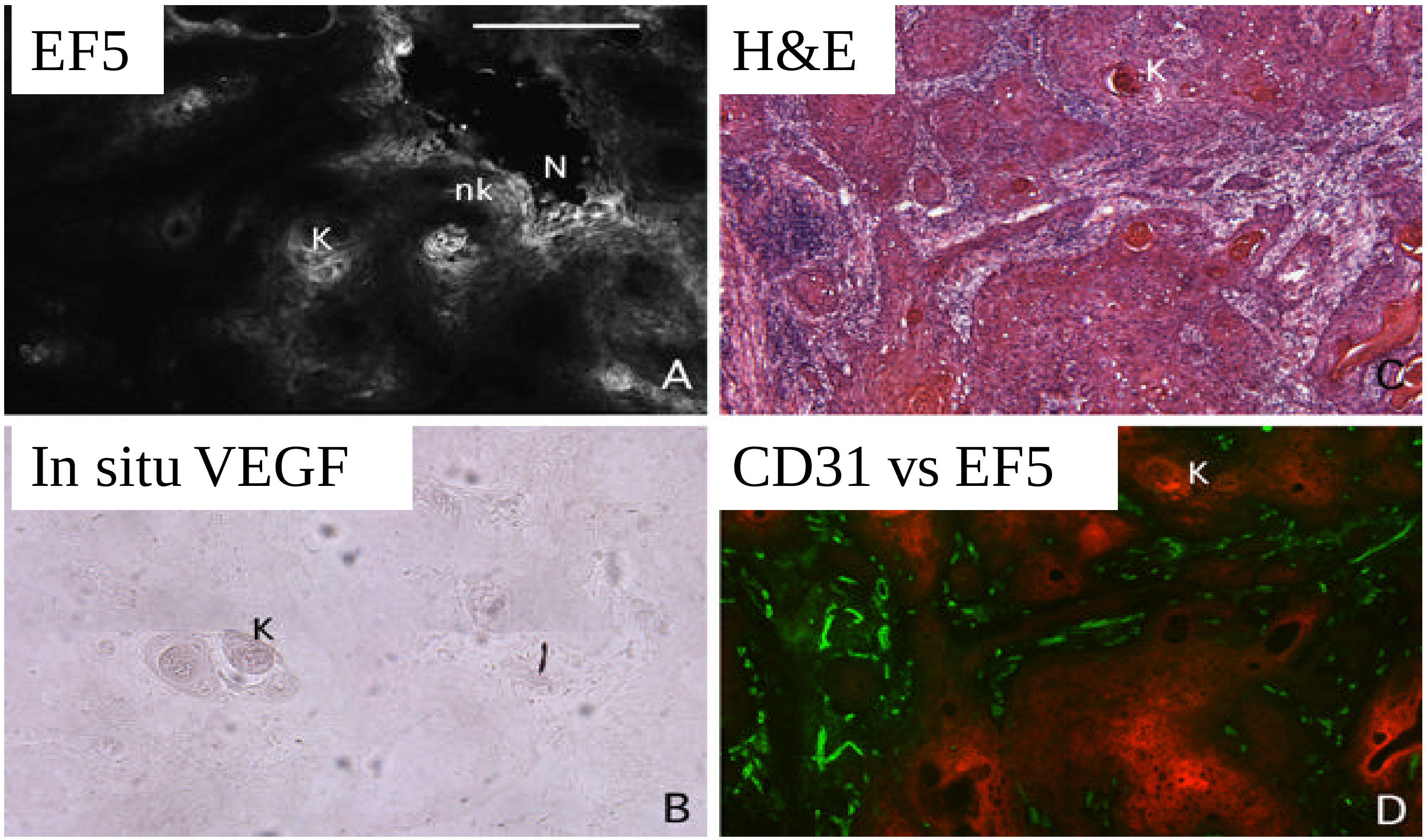
From Raleigh *et al.*, Cancer Research 58:3765, 1998

Co-localization EF5 / VEGF mRNA - Soft Tissue Sarcoma



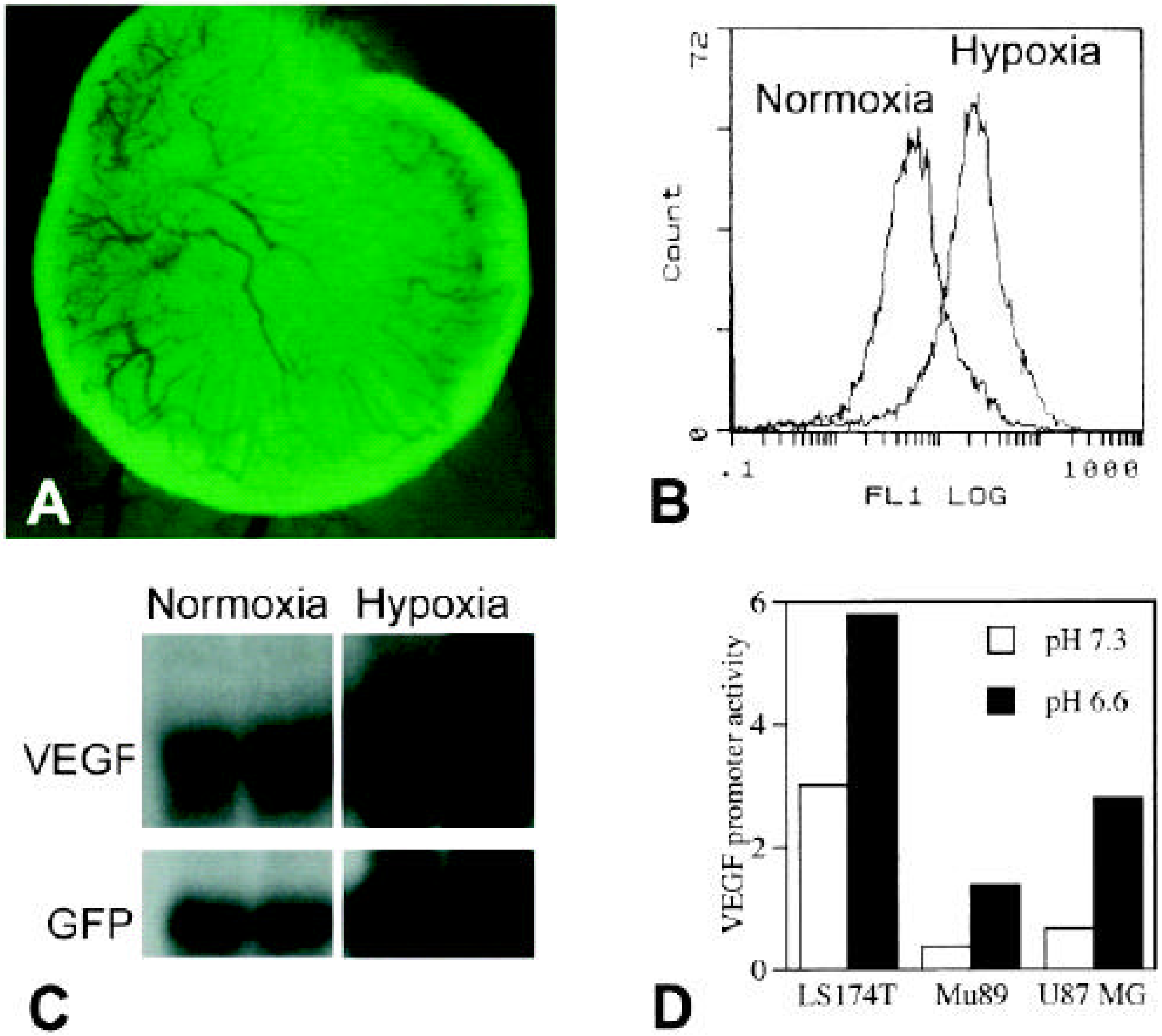
Data from: Evans and Koch

High EF5 binding / keratin without VEGF: Squamous Cell Ca



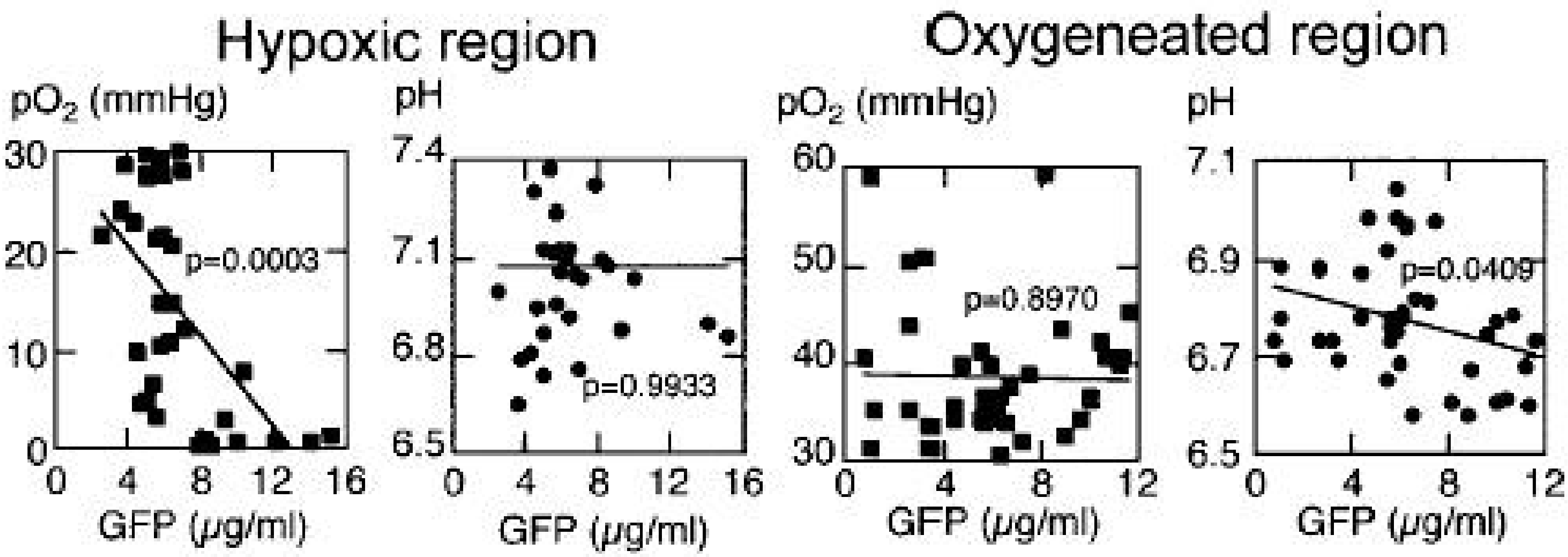
From: Evans and Koch

pO_2, pH effect on VEGF Expression



Fukumura *et al.*, 2001

Proposed independence between pH and pO_2 in controlling VEGF expression



Fukumura *et al.*, 2001

Questions regarding tumor angiogenesis

- What is the relation between hypoxia and angiogenesis?
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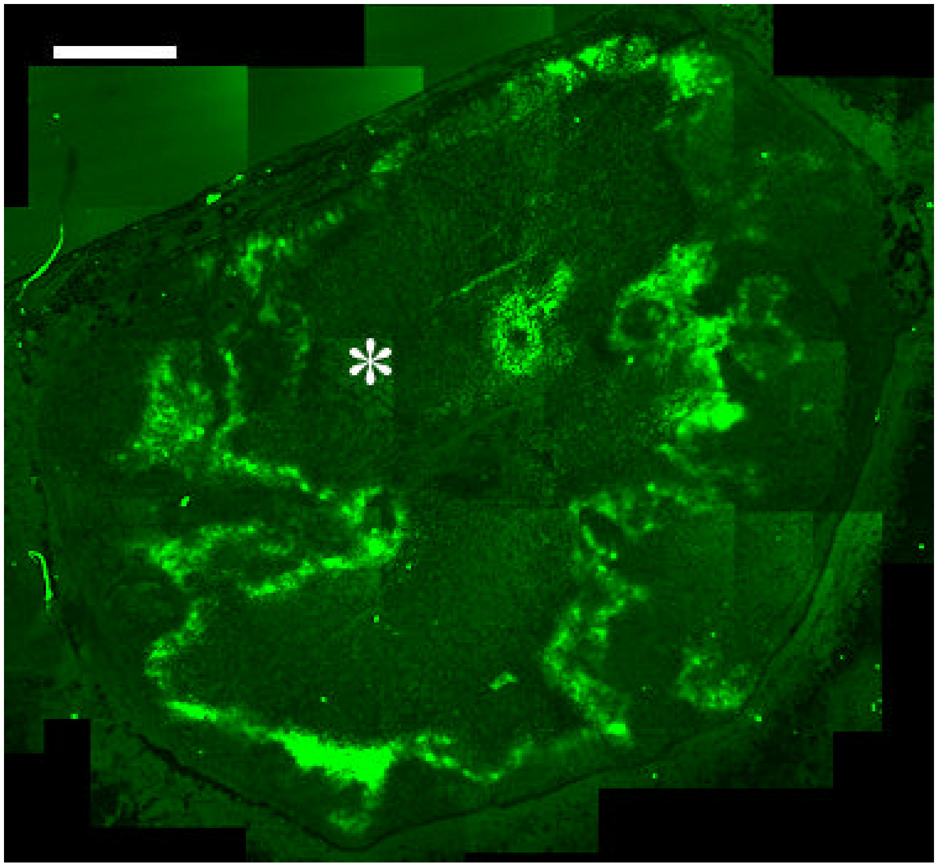
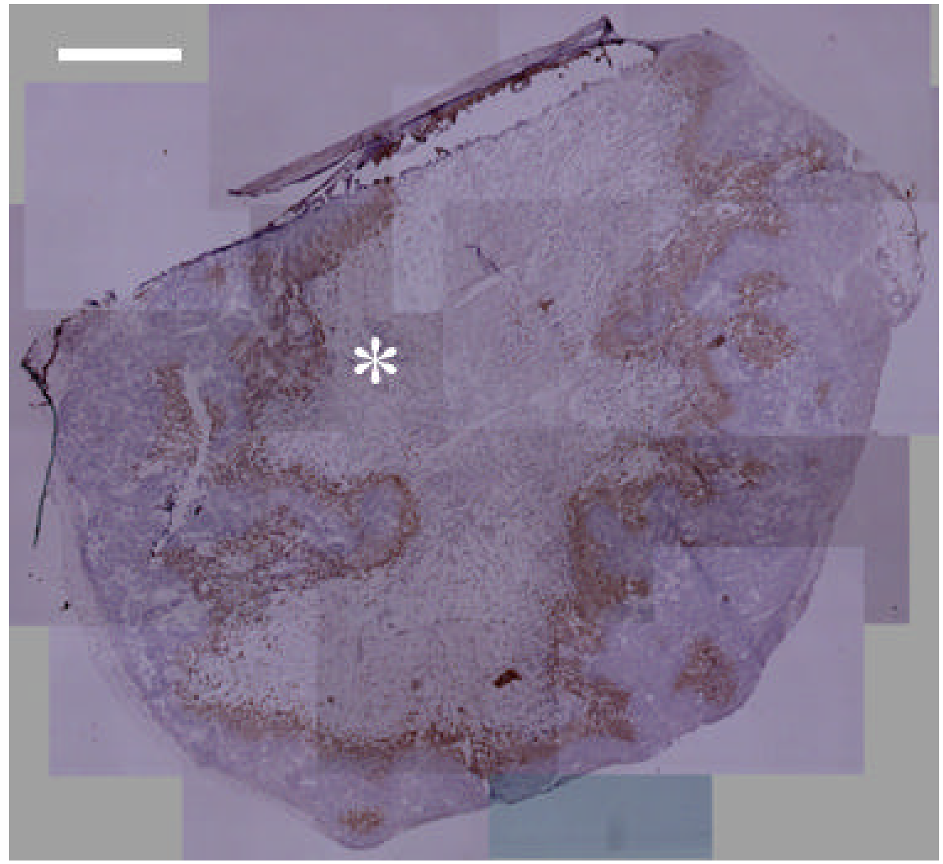
Hif-1 promoter vs. hypoxia marker (pimonidazole): HCT 116 Xenograft



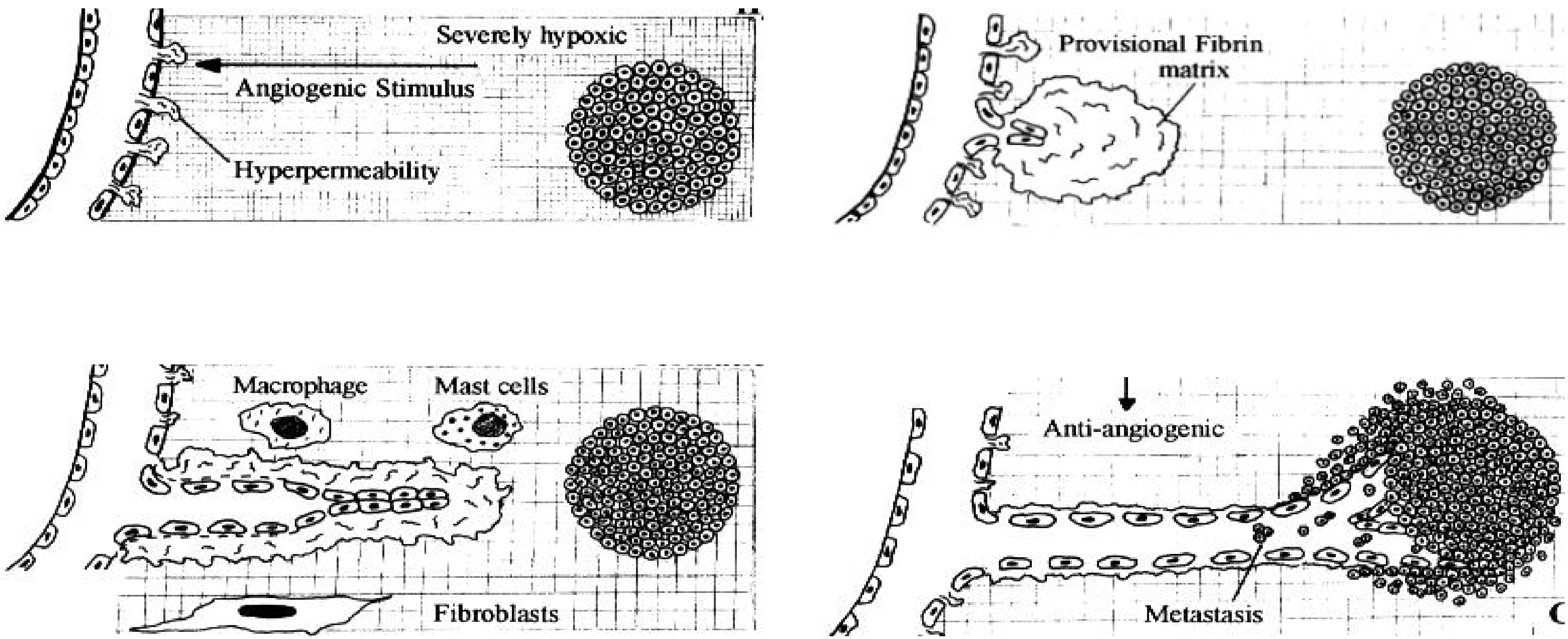
Hif-1 EGFP reporter gene



Pimonidazole binding

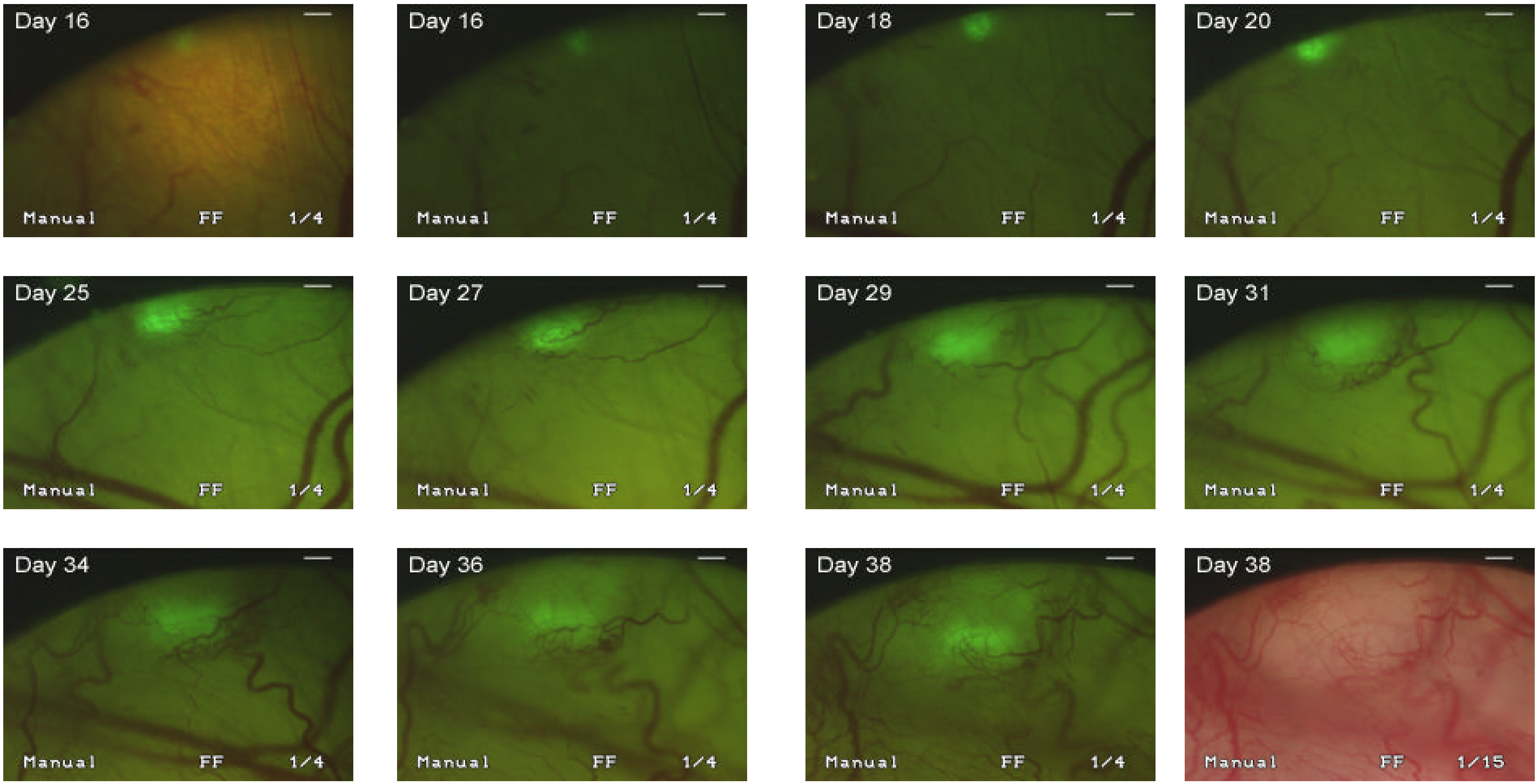
Cao and Dewhirst, unpublished

Classic Steps of TumorAngiogenesis



Courtesy of I. Haroon

Serial Observation of Hif-1 -GFP and Angiogenesis in Window Chamber

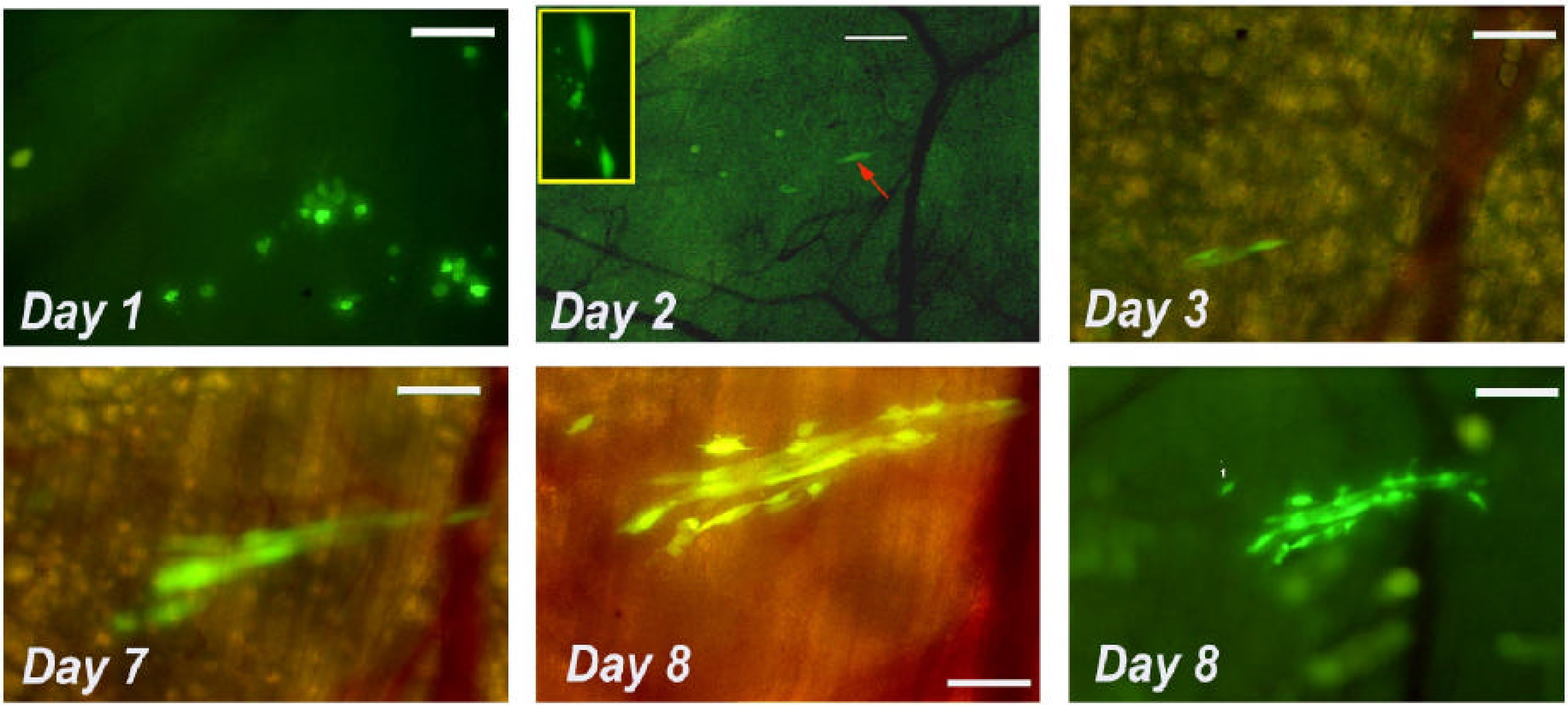


Bar: 200 mm

HCT 116 Colon Carcinoma Xenograft

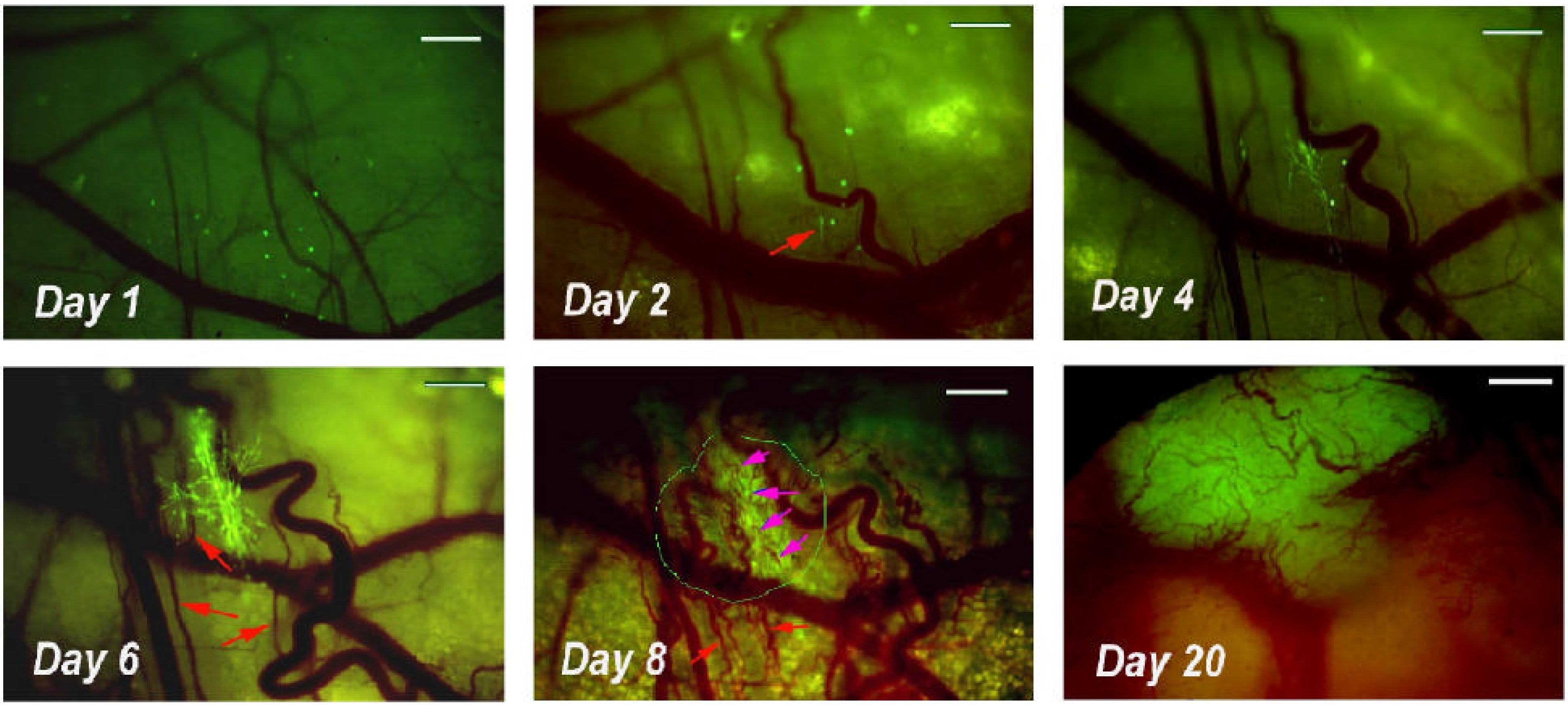
Y. Cao, C.Y. Li, M. Dewhirst, unpublished

Early Angiogenesis: Chemotactic proliferation of tumor cells toward host vessels



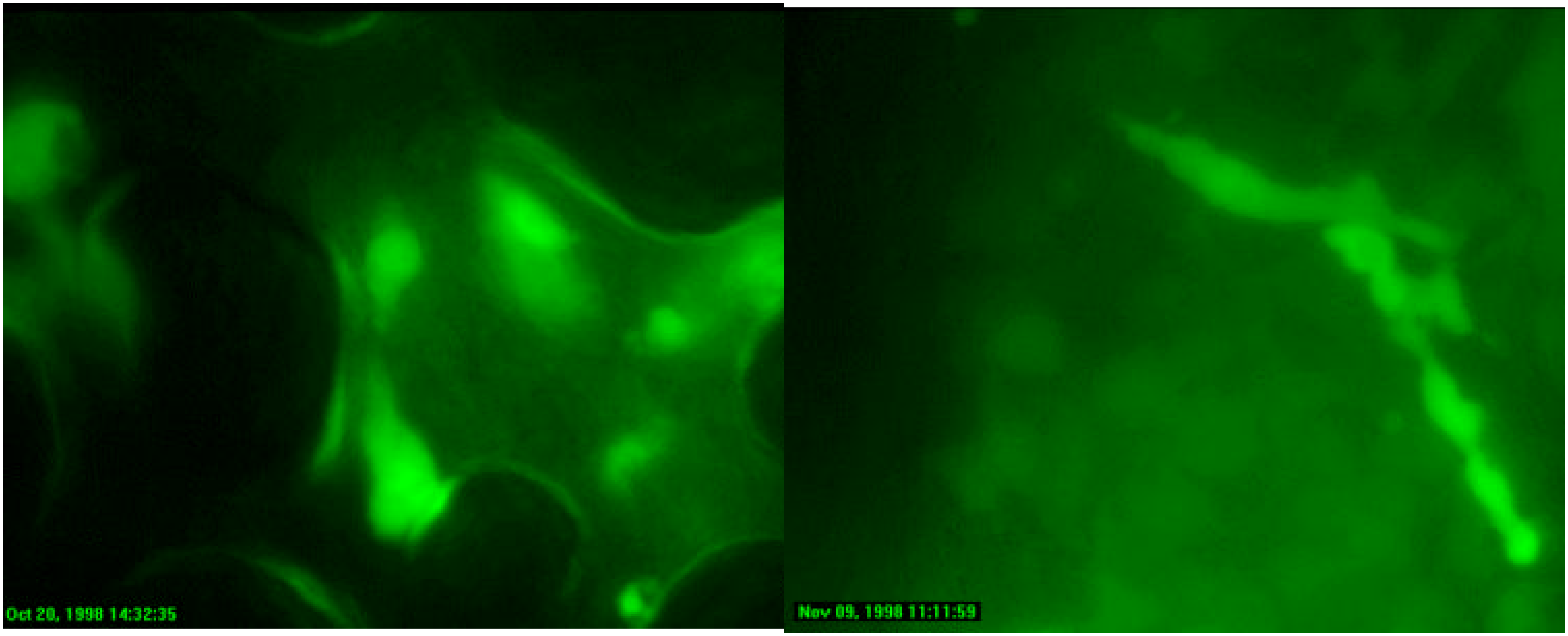
4T1 Cell Line: Data from C.Y. Li, JNCI, 2000

Early Tumor Angiogenesis: Angiogenesis onset at 200-cell stage



4T1 Cell line

Pulmonary Metastasis involves intravascular proliferation before angiogenesis



Courtesy of Ruth Muschel

Features of Early Angiogenesis

- Hif-1 upregulation @ very early stage (<200μm tumor, <50 cells)
- Onset of angiogenesis very early (200-300 cell stage)
- Vascular remodeling (regression) prominent
  - Wound like behavior

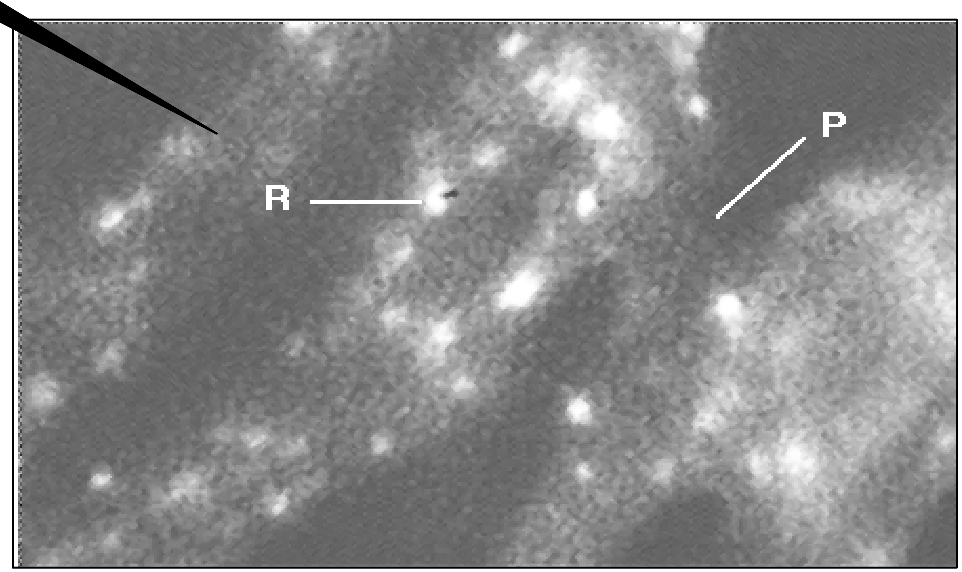
Questions regarding tumor angiogenesis

- What is the relation between hypoxia and angiogenesis?
- Is early angiogenesis driven by hypoxia?
- Is vascular remodeling driven by hypoxia?
- Is there a role for Reactive Oxygen Species in Tumor Angiogenesis?

Characterization of intermittent flow and pO_2 - window chamber preparation

3-6μm Microelectrode

TRITC liposomes
DII labeled RBCs

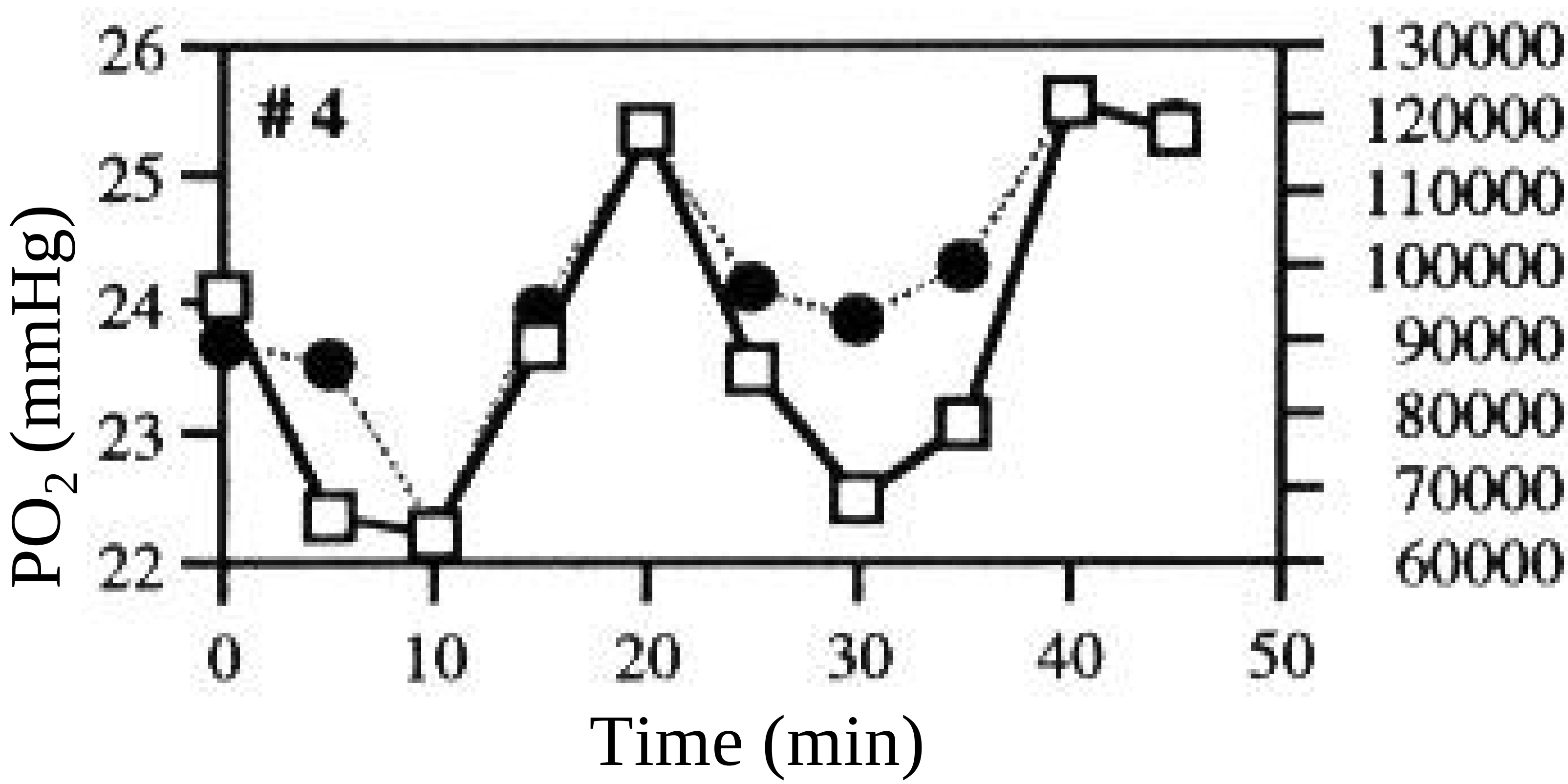


<u>Frequency Data : Vascular Stasis</u>

% with complete stasis:	5%
Duration of stasis:	<1min
Collapse:	none
Vasoconstriction:	none
Leukocyte occlusion:	none

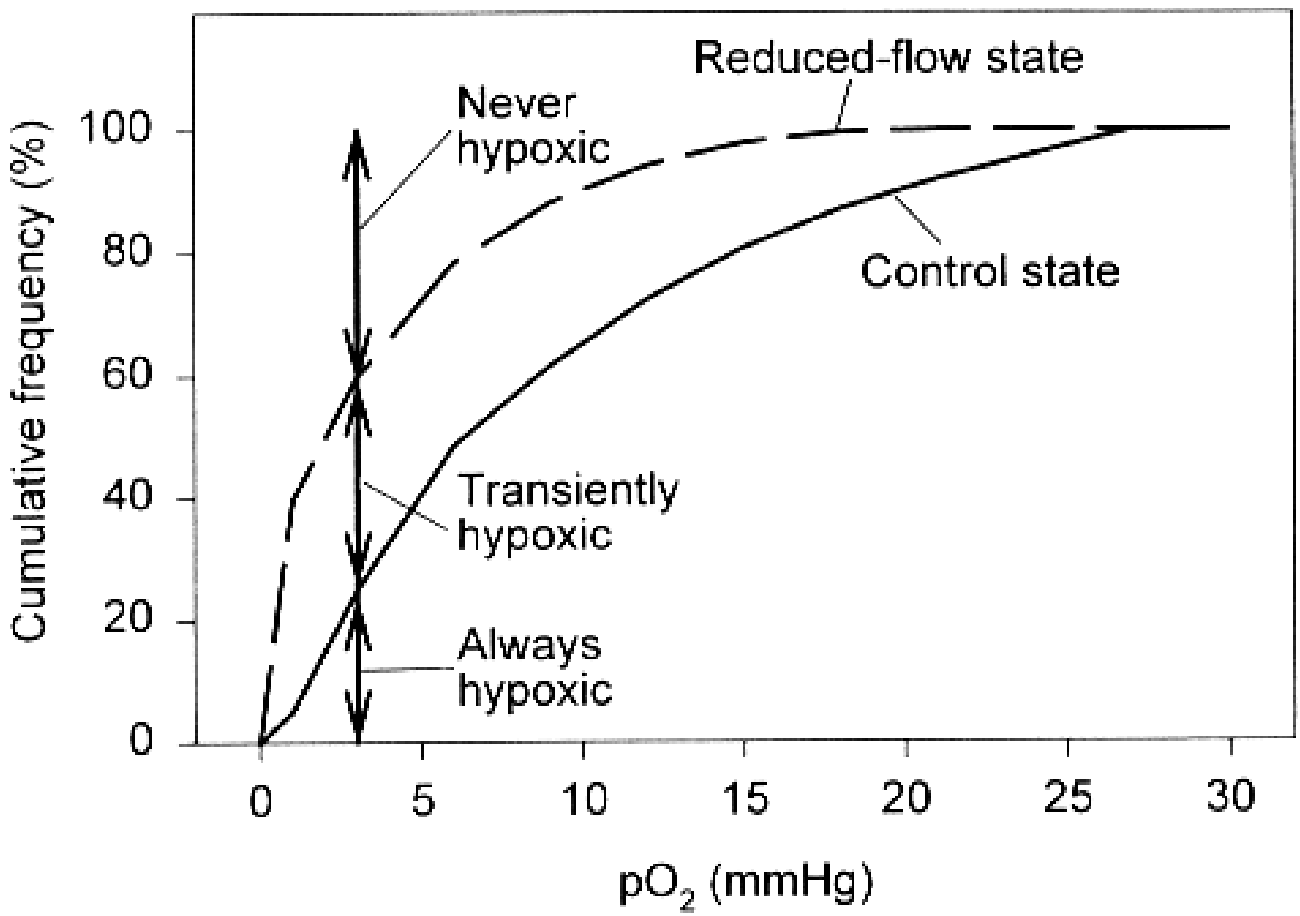
From: Dewhirst *et al.*, Br. J Cancer, 74:S247, 1996

Red cell flux relates to perivascular pO_2

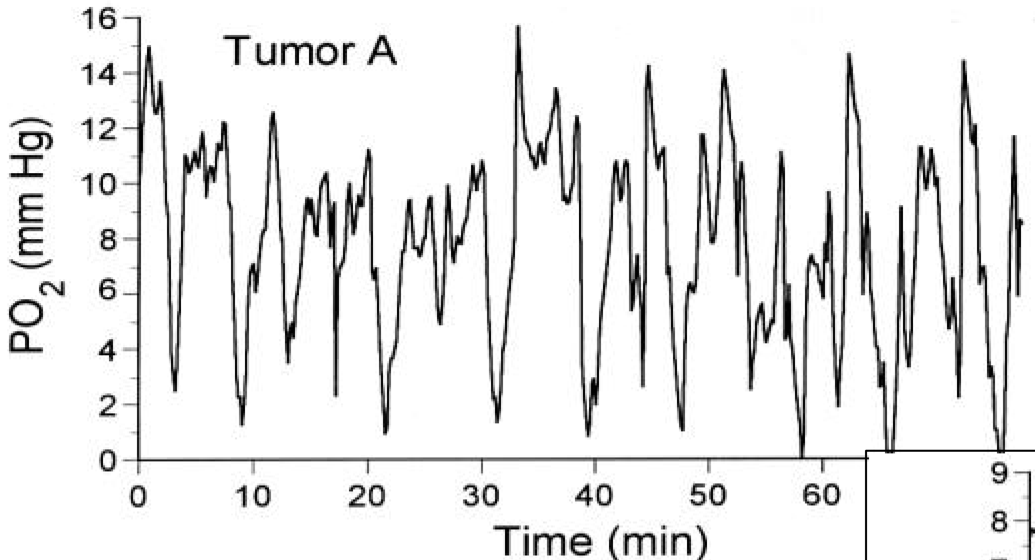


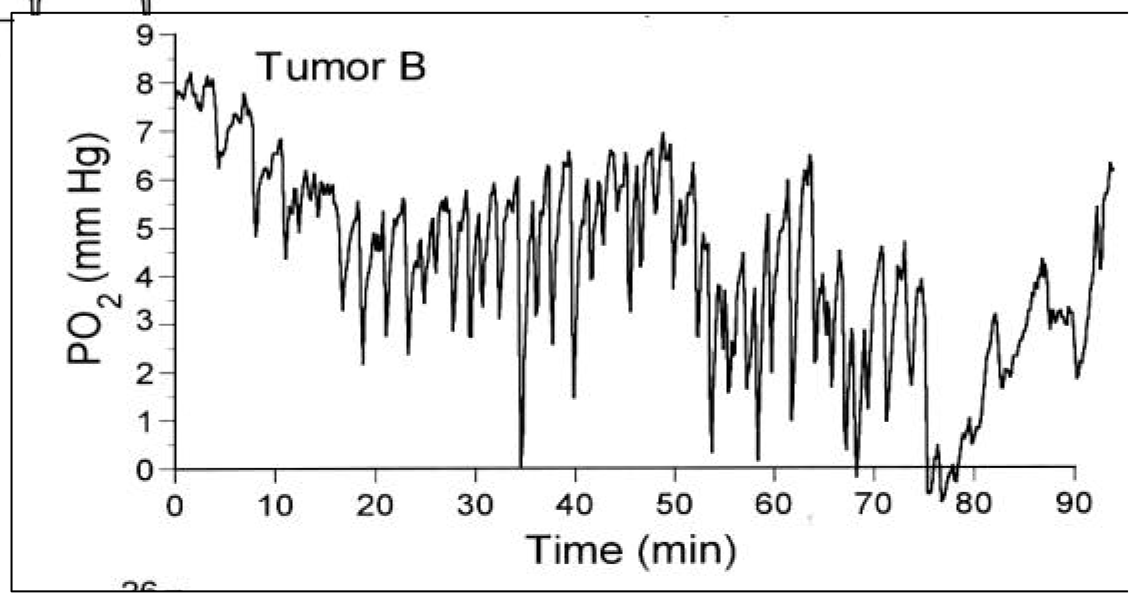
From Kimura *et al*., 1996

Projected effect of RBC flux variation (2x change) on tumor hypoxia



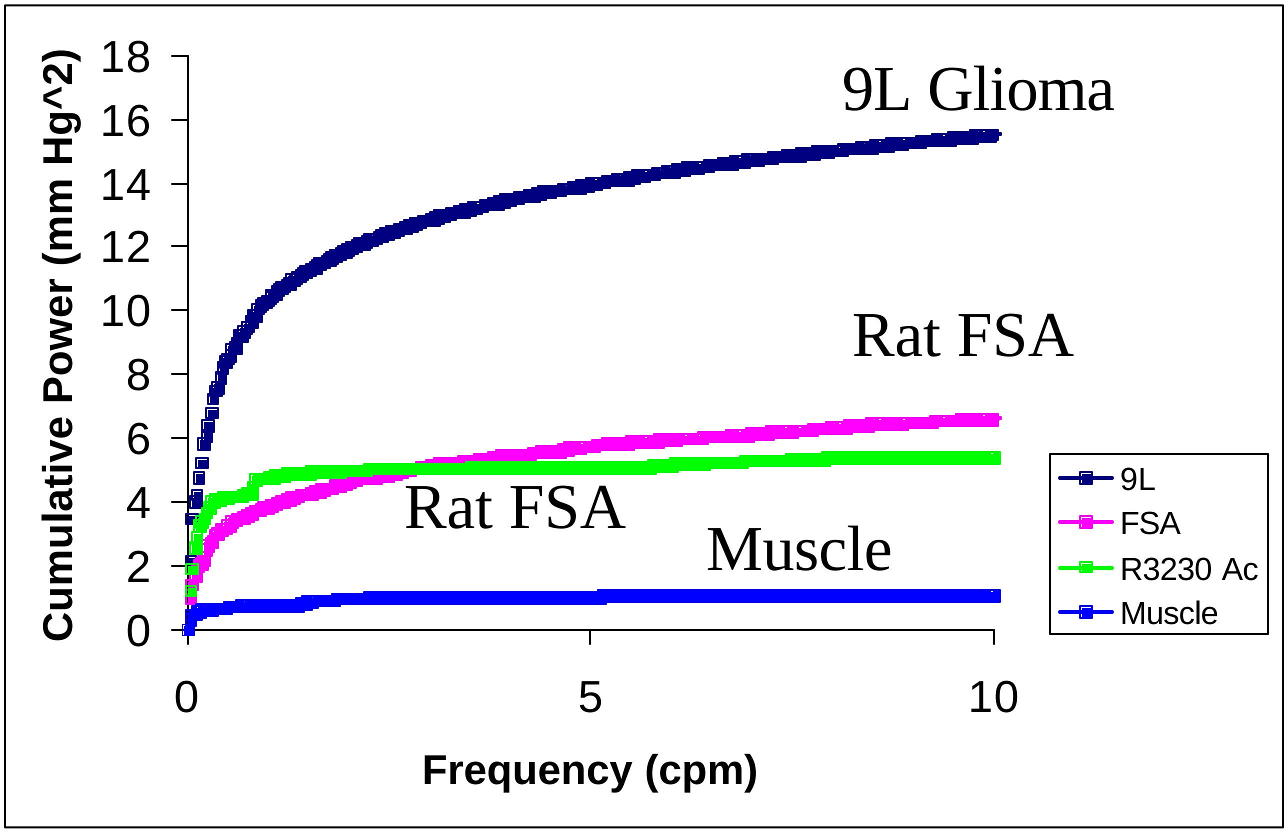
Intermittent hypoxia as potential source of ROS



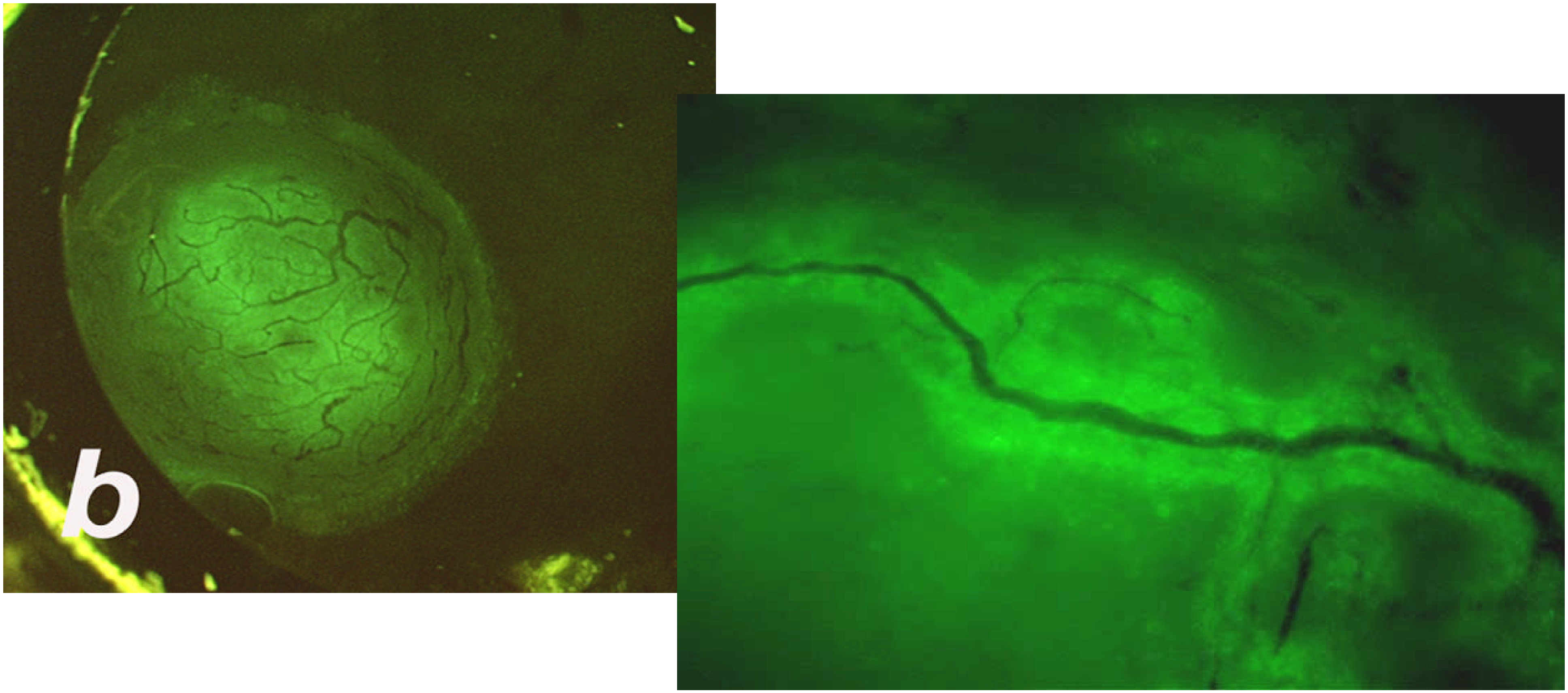


R3230 Ac Tumor Line
10µm tip electrodes
Braun, AJP, 1999

Fourier Transform Analysis: Comparison of three tumors vs. muscle

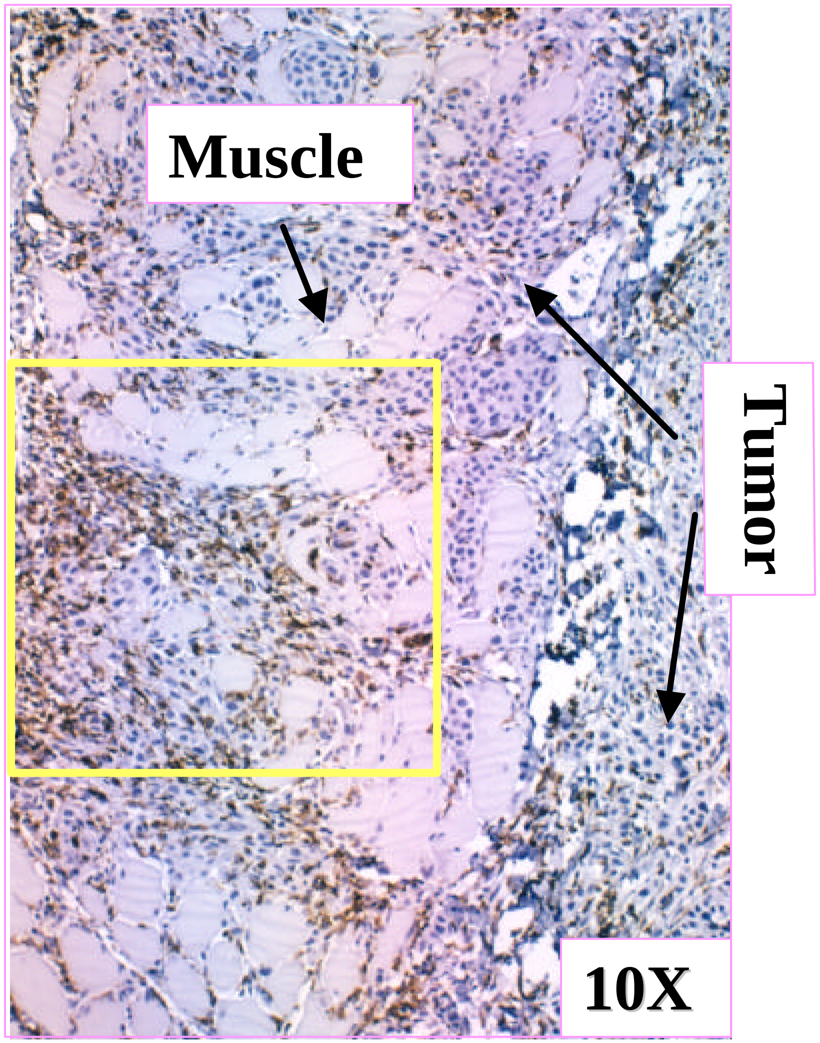


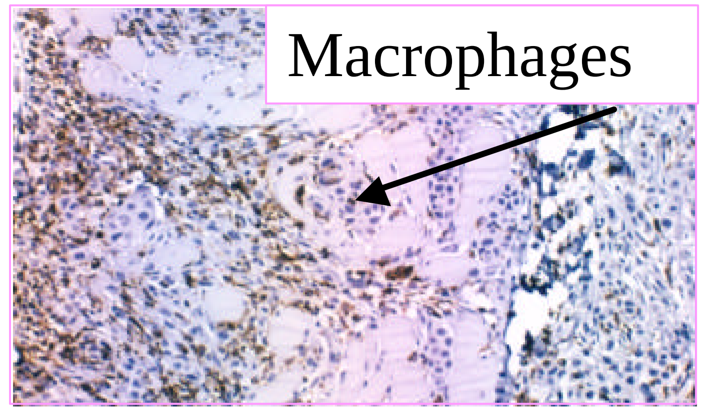
Is spatial variation of HSP70-GFP caused by ROS?

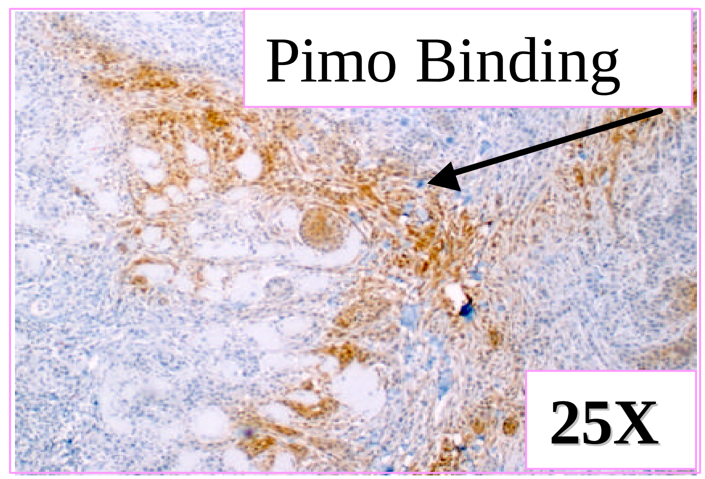


R3230Ac cell line: C.Y. Li, S. Shan, M. Dewhirst- Unpublished data

Co-localization of hypoxia / macrophages: Active angiogenic foci







R3230Ac tumor: Z. Haroon

Potential Source of Reactive Oxygen Species - Tumor

- **Activated Macrophages**
- **Unstable perfusion**
  - **Very frequent in some tumors**

ROS may be important regulator of angiogenesis in tumors

Conclusions: Normal Tissues

- Hypoxia can exist without angiogenesis
- ROS important in pathologic angiogenesis
- Hypoxia may regulate vascular regression in wounds

Tumor vs. wound angiogenesis

- **Remodeling of tumor vasculature mimics wound regression that occurs with hypoxia**
- **ROS may play important role in angiogenesis regulation in tumors**
  - **Macrophages may contribute to hypoxia in manner similar to wound healing**
  - **Intermittent flow may also contribute to oxidative stress**

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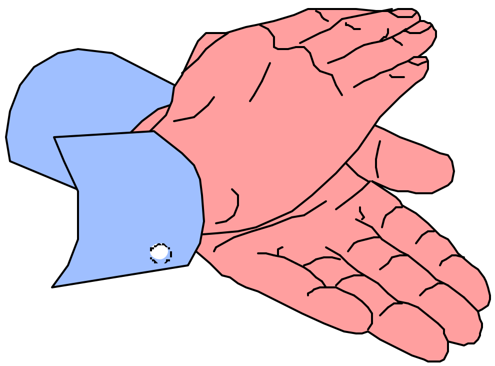

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